UNIVERSIDAD COMPLUTENSE DE MADRID
FACULTAD DE CIENCIAS BIOLÓGICAS

TESIS DOCTORAL

Intervenciones nutricionales en modelos de envejecimiento prematuro y cronológico

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

Caroline Hunsche

Directora

Mónica de la Fuente del Rey

Madrid

UNIVERSIDAD COMPLUTENSE DE MADRID

FACULTAD DE CIENCIAS BIOLÓGICAS

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TESIS DOCTORAL

CAROLINE HUNSCHE **DRA. MÓNICA DE LA FUENTE DEL REY**

Madrid, 2019

UNIVERSIDAD COMPLUTENSE MADRID

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La realización del presente trabajo se ha llevado a cabo gracias a la ayuda económica de los proyectos de investigación BFU2011-30336 del actual Ministerio de Ciencia, Innovación y Universidades (MICINN), PI15/01787 del Fondo de Investigación en Salud (FIS) del ISCIII-FEDER de la Unión Europea, de la Red de Envejecimiento y Fragilidad (RETICEF) (RD12/0043/0018) del Instituto de Salud Carlos III, y del grupo UCM-CM: Envejecimiento, Neuroinmunología y Nutrición (ENEROINN) (N° 910379).

Caroline Hunsche obtuvo la concesión de una beca del Consejo Nacional de Desarrollo Científico y Tecnologico (CNPq) del Brasil (248447/2013-0) del 01/06/2014 al 30/09/2018.

ACKNOWLEDGEMENTS

Undertaking this Ph.D. has been a truly challenging, but at the same time, rewarding experience. This would not have been possible without the support and generosity of many people.

I would like to express my deepest gratitude and sincere thanks to my Ph.D. supervisor Mónica De la Fuente for her generous guidance, endless support and encouragement throughout my research studies. I joined her laboratory as a M.Sc. student and later became a Ph.D. student. She gave me the opportunity to join her laboratory and introduced me to the interesting field of aging research and taught me a lot, not only the theoretical concepts, but also the different aspects of being a researcher. Her scientific knowledge, passion, motivation and patience helped me to develop new skills and ideas.

I owe my warmest gratitude to the friends that I made during this journey. Oskarina for her enormous generosity and kindness - I will always remember her warm welcome when I first arrived in the laboratory. Irene for her happiness and enthusiasm, and for her great support during this entire process. Carmen for having such a good heart. I also owe my warmest thanks to Cristina, Edu, Lucia, Virginia, Ianire, Julia, Garrido, Nuria, Noemi, Estefania, and Hikaru for their great support and kindness.

I am very grateful to all those at the department of Animal Physiology, especially to Ana, Teresa Peláez and Teresa Rodríguez, who were always so helpful and kindness.

I am also very grateful to my mother in law Nelci, who is a scientific researcher, for the great advices throughout my studies.

I would like to extend my deepest gratitude to my parents Wilson and Rosani, siblings Thales and Raquel, grandmother Selmira and uncle Paulo for their unconditional support, encouragement and love. My mum, for being the kindest person in the world, and my dad, for always being there when I needed the most.

Finally, the very special thanks are reserved to my beloved husband, Rodrigo, who has been always by my side, for his love, support and understanding over the years. And to my beloved daughter Livia who has made me stronger and more fulfilled than I ever imagined. This thesis is dedicated to them.

CONTENTS

RESUMEN

INTERVENCIONES NUTRICIONALES EN MODELOS DE ENVEJECIMIENTO PREMATURO Y CRONOLÓGICO

En el envejecimiento tiene lugar un deterioro funcional progresivo de todos los sistemas fisiológicos del organismo, especialmente de los reguladores, el sistema nervioso, el endocrino y el inmunitario, lo que conlleva la pérdida de homeostasis y el consecuente aumento de morbilidad y mortalidad. Según la teoría de la oxidación-inflamación del envejecimiento, el deterioro al envejecer en los sistemas homeostáticos es consecuencia del estrés oxidativo e inflamatorio crónico del organismo, y en el que el sistema inmunitario está muy implicado. De esta forma, se propone que este sistema que tiene que producir oxidantes e inflamación para llevar a cabo sus funciones, puede, si no está bien regulado, condicionar la velocidad de envejecimiento de cada individuo, siendo un marcador del estado de salud y predictor de longevidad.

El envejecimiento es un proceso fisiológico que puede ser acelerado o retardado por factores ambientales. Dada la gran longevidad del ser humano se requieren animales de experimentación, como los ratones, con una esperanza de vida corta, en los que sea posible estudiar las consecuencias de un envejecimiento acelerado y de las intervenciones que puedan conseguir una mayor longevidad saludable. Nuestro grupo de investigación ha caracterizado modelos animales de envejecimiento prematuro o acelerado. Uno de dichos modelos es la obesidad, en el que se ha sugerido la presencia de una inmunosenescencia prematura.

Por otro lado, la leptina, una hormona pleiotrópica que, aunque relacionada típicamente con la obesidad, tiene una gran variedad de funciones fisiológicas en el organismo, experimenta en roedores una elevación de sus niveles en los días 4 a 14 postnatales. Estudios previos han apuntado que esta elevación neonatal de la leptina tendría una función neurotrópica sobre el hipotálamo en desarrollo. Sin embargo, poco se sabe acerca del papel que ese aumento de leptina podría tener en el sistema inmunitario, en su capacidad funcional y su estado redox/inflamatorio.

Entre los factores de estilo de vida que pueden retrasar el deterioro de los sistemas reguladores ocasionado tanto por un envejecimiento prematuro, como puede ser la obesidad, o por el propio envejecimiento cronológico, se encuentra la nutrición. Los ácidos grasos monoinsaturados, los poliinsaturados derivados de la serie n-3, tales como el ácido eicosapentaenoico (EPA) y ácido docosahexaenoico (DHA), y los probióticos son eficaces en modular funciones del sistema inmunitario y, además poseen acciones antioxidantes.

Por todo ello, los objetivos planteados en la presente tesis han sido los siguientes:

1er objetivo: Estudiar, en ratones, los efectos de la obesidad inducida por dieta en el comportamiento, la función inmunitaria y el estado redox/inflamatorio en diferentes edades, así como su relación con la longevidad.

2º objetivo: Estudiar los efectos del bloqueo de la leptina neonatal en la función inmunitaria y el estado redox/inflamatorio en ratas machos y hembras en diferentes edades.

3er objetivo: Estudiar en ratones adultos, obesos por dieta, los efectos de la suplementación nutricional con ácido hidroxioléico (2-OHOA) y con EPA y DHA en la función inmunitaria y el estrés oxidativo, así como en la longevidad.

4º objetivo: Estudiar, en ratones viejos, los efectos de la suplementación nutricional, durante diferentes períodos, con una leche fermentada que contiene probióticos, en el comportamiento, la función inmunitaria y el estrés oxidativo, así como en la longevidad.

Los **resultados** del 1er objetivo permiten **concluir** que la ingestión de dieta rica en grasa en la adolescencia de ratones hembras supone llegar a la edad adulta con una obesidad que se asocia con una inmunosenescencia prematura y un estrés oxidativo-inflamatorio. Este deterioro es agravado durante el envejecimiento, aunque ya sólo ingieran dieta estándar, llegando a la vejez con un estado inmunitario y redox peor que los cronológicamente viejos que nunca fueron obesos. Cuando la obesidad es inducida ya en la edad adulta aparece un deterioro del comportamiento, de la función inmunitaria y del estado redox en ratones machos y hembras de edad adulta tardía. Se observan diferencias por el sexo en algunos de los parámetros comportamentales, inmunitarios y de

estado redox, siendo los machos más afectados que las hembras. La inmunosenescencia acelerada que presentan estos animales es agravada en la vejez. Estos resultados confirman el envejecimiento prematuro y acelerado que aparece como consecuencia de la obesidad inducida por dieta en la adolescencia y en la edad adulta, respectivamente, causando un mayor deterioro la primera.

En cuanto al 2º objetivo, los resultados indican que las ratas machos y hembras tratadas con un antagonista de la leptina entre los días 5-9 postnatales, presentan un deterioro de la función inmunitaria en el bazo y del estado redox/inflamatorio en el bazo, hipotálamo y tejido adiposo durante la adolescencia. El deterioro en la función inmunitaria y en el estado redox del bazo fue también observado en la edad adulta por ratas machos y hembras sometidas al mismo tratamiento postnatal. Estos resultados corroboran el papel de la leptina neonatal en el establecimiento y mantenimiento de la función inmunitaria y del estado redox/inflamatorio en ratas machos y hembras.

En lo referente al 3er objetivo, los resultados muestran que en los ratones hembras adultas que son obesas por ingerir una deta rica en grasa durante la adolescencia, y que además del deterioro de la función y estado redox en leucocitos, ya observado en el objetivo primero, tienen un deterioro redox en el cerebro, hígado, pulmones y riñones, la ingestión de 2-OHOA y la combinación de (EPA y DHA), durante el final de la adolescencia, permite revertir tales deterioros. Así, esta suplementación puede ser una buena intervención nutricional para restaurar la función inmunitaria y el estado redox en ratones obesos, y consecuentemente promover una longevidad saludable.

En el 4º objetivo, los resultados indican que, en ratonas viejas, la suplementación nutricional con una leche fermentada con probióticos durante 1, 2 y 4 semanas revierte el deterioro comportamental, de función inmunitaria y de estrés oxidativo típicos de su edad. Esa suplementación tiene además una acción directa en el funcionamiento de las células inmunitarias. Por lo tanto, estos resultados indican que la ingestión de probióticos puede ser una buena intervención nutricional para promover una longevidad saludable.

ABSTRACT

NUTRITIONAL INTERVENTIONS IN MODELS OF PREMATURE AND CHRONOLOGICAL AGING

Aging is characterized by a progressive deterioration of all physiological systems of the organism, especially those of the regulatory systems namely the nervous, endocrine and immune systems, leading to loss of homeostasis, and consequently increased morbidity and mortality. According to the oxidation-inflammation theory of aging, the age-related deterioration of the regulatory systems is the result of the chronic oxidative and inflammatory stresses. This theory also proposes that the immune system, due to its capacity of producing oxidant and inflammatory compounds in order to carry out its defensive role, if not well regulated, could be involved in the rate of aging. Thus, the immune system has been suggested as a marker of health status and predictor of longevity.

Aging is a physiological process that can be accelerated or delayed by environmental factors. Experimental animals such as rodents, which have a shorter longevity than humans, have been used to study the consequences of an accelerated aging, as well as interventions aimed to achieve a healthy aging. Our research group has been characterizing murine models of premature and accelerated aging. Along with these models, obesity has been proposed as a possible model of premature immunosenescence.

Leptin, a pleiotropic hormone, which has been classically related to obesity, has shown a great variety of physiological functions in the organism. This hormone experiences an elevation of its levels in postnatal days (PND) 4 to 14 in rodents. Previous studies suggest that this neonatal increase of leptin levels would have a neurotropic function in the developing of hypothalamus. However, little is known about the specific physiological role of this increase of neonatal leptin regarding the immune system, such as immune function and redox/inflammatory state.

Among lifestyle factors, nutrition has been proposed as very relevant to delay the deterioration of the regulatory systems caused by obesity, a possible model of premature aging, and

by chronological aging. Monounsaturated fatty acids, polyunsaturated fatty acids derived from n-3 series (eicosapentaenoic acid and docosahexaenoic acid) and probiotics are effective in modulating immune system functions and also have antioxidant and anti-inflammatory properties.

Taking all of this into account, the following objectives of the present thesis are:

1st objective: To study the effects of diet-induced obesity onset on behaviour, immune function and redox/inflammatory state at different ages, as well as on the life span of mice.

2nd objective: To study the effects of the blockage of the neonatal leptin surge (PND5-9) on immune function and redox/inflammatory state of male and female rats at different ages.

3rd objective: To study the effects of the dietary supplementations with 2-OHOA or with the combination of n-3 fatty acids (EPA and DHA) on immune function and redox state of adult female diet-induced obese mice, as well as on their life span.

4th objective: To study the effects of the dietary supplementation with fermented milk containing probiotics for different periods of time on behaviour, immune function and redox state of old mice, as well as on their life span.

The **results** obtained from the 1st objective allow the **conclusion** that the high-fat intake during adolescence produces a state of obesity, which is associated with premature immunosenescence and oxidative-inflammatory stresses in adult female mice. Old female diet-induced (DIO) mice, although ingesting a standard diet during their aging process, show higher deterioration of immune function and oxidative stress in comparison with non-DIO mice of the same chronological age. In addition, the late adulthood diet-induced obesity onset results in impaired behaviour, immune function and redox state of middle-aged male and female mice. Sex differences are found in some of the parameters of behaviour, immune function and redox state, males being more affected than females. These female animals show accelerated immunosenescence, which are partially aggravated with aging. These results confirm the state of premature and accelerated aging, as a consequence of the early and late adulthood diet-induced obesity onsets, respectively.

With regards to the 2nd objective, the results indicate that male and female rats treated with the leptin antagonist from PND 5 to PND 9 show impaired immune function and redox/inflammatory state in the spleen, hypothalamus and adipose tissue during adolescence. In addition, at adulthood, the impairment of immune function and redox state is also observed in male and female rats treated with the same leptin antagonist. These results corroborate the important role of the neonatal leptin in the establishment and maintenance of a proper immune function and redox/inflammatory state in male and female rats.

Regarding the 3nd objective, the results indicate that adult female DIO mice, as a consequence of being fed a high-fat diet during their adolescence, show impaired immune function and redox state in leukocytes, as previously observed in the first objective. Additionally, these animals exhibit altered redox state in the brain, liver, lungs and kidneys. Nevertheless, the dietary supplementations with monounsaturated (2-OHOA) or with the combination of n-3 polyunsaturated fatty acids (EPA and DHA) revert these impairments in adult female DIO mice. The results indicate that these supplementations could be a good nutritional intervention to restore an appropriate immune function and redox state in DIO mice, and consequently promote a healthy aging.

The results obtained in the 4th objective indicate that the dietary supplementation with fermented milk containing probiotics for 1, 2 and 4 weeks reverts the impaired behaviour, immune function and redox state in old female mice. The improvement of the immune cell functions seems to be due to the direct action of probiotics. Therefore, these results indicate that the ingestion of probiotics could be a good nutritional intervention to promote a healthy aging.

1. INTRODUCTION

1.1. PROCESS OF AGING

1.1.1. Aging of the population

The global aging of the population is increasing at an unprecedented rate. The world population aged 65 years or older is projected to double from about 506 million in 2008 to 1.3 billion by 2040, accounting for 14% of the total population in the world. Although developed countries have relatively high proportions of older people and the longest life expectancies, the most rapidly increase has been experienced by developing countries (Cauley, 2012). The aging of the population is being driven mainly by declines in fertility and increases in longevity. In addition, the transition of the dominant causes of death and illness from infectious diseases to non-communicable chronic diseases has positively impacted the life expectancy. Although this transition reflects substantial improvements in overall health, new challenges are arising. There is an increasing number of elderly people suffering from chronic diseases, including cancer, cardiovascular diseases, type 2 diabetes, obesity, and Alzheimer´s disease. These chronic diseases are associated with disability, poor health status and loss of autonomy living (Foreman *et al*., 2018). Therefore, according to World Health Organization´s (WHO) report on aging and health, the adoption of lifestyle strategies, such as physical activity and good nutrition, is a mandatory achievement in order to prevent or delay many of these chronic diseases, and consequently to reach advanced ages in healthy conditions (WHO, 2015).

1.1.2. Definition of aging

Aging is associated with a progressive accumulation of unrepaired molecular and cellular damage (Kirkwood, 2005). The accumulation of this damage over time leads to a gradual deterioration of physiological functions and to a lower ability to adaptively react to changes and maintain homeostasis (or homeodynamics) (De la Fuente, 2018a). The concept of homeodynamics refers to the fact that the internal milieu of biological systems is not permanently fixed or static, but

rather is dynamically reorganized in response to internal or external challenges (such as stress) in order to preserve the functional capacity of the systems (Rattan, 2006). For instance, with aging there is a general decreased functional capacity of elderly people to endure extreme temperatures, infections or other situations in which stress occurs. If the principal characteristic of a healthy organism is to maintain the functional homeostasis (or homeodynamics) at all biological levels of organization, with age this fails. Although aging in itself cannot be considered a disease, it strongly increases the risk of illness and death (De la Fuente and Miquel, 2009).

According to Strehler (1977), aging may be defined following four rules (1) it is universal (nearly all species of animals including the metazoans with sexual reproduction suffer from aging), (2) progressive (the rate of aging is similar after adult age throughout the rest of life) (3) intrinsic (the causes of aging are initiated endogenously), and (4) deleterious (aging is harmful to the individuals since it results in their death).

1.1.3. Mean and maximum longevity

The terms life span and longevity, which are used interchangeably, refer to the period of time between birth and death of an individual. The mean and maximum longevity values provide useful measures to study the process of aging. The mean longevity can be defined as the mean age at death of the population who born on the same data (Pletcher *et al*., 2000). The mean longevity is mostly determined by environmental agents, including lifestyle factors (around 75%) and to a lesser proportion by genetic factors (around 25%) (Barja, 2013; De la Fuente and Miquel, 2009). In contrast, the maximum longevity, which is the age reached by the longest-lived member of a species, is stable and cannot be extended (Balcombe, 2001). For instance, in humans, the maximum longevity should be limited to around 125 years (the oldest person who ever lived was Jeanne Calment from France who reached 122 years old and died in 1997) and, in laboratory mouse strains, to around 3 years old (de Beer *et al*., 2017; De la Fuente, 2008; Marck *et al.*, 2017).

1.1.4. Biological age

The process of aging is very heterogeneous across the human population. The interindividual variability of aging arises from variations in the exposure to environmental stressors as well as the innate ability of the body to cope with these stressors (Levine, 2013). Although the prevalence of functional impairments, illness and mortality rises with age, many people reach old age maintaining good health, active lifestyles and autonomy living (Lowsky *et al*., 2014). Therefore, chronological age appears to fail in providing an accurate indicator of the rate of aging (Finkel *et al*., 1995; Karasik *et al*., 2005). The concept of biological age has emerged as a relevant method to quantify the functional capacity and health status of an individual in comparison with others of the same chronological age (Kim and Jazwinski, 2015). The assessment of biological age may help to recognize individuals at high risk for age-related disorders and mortality independent of chronological age (Levine, 2013). However, the exploration and validation of markers that quantify biological age remain largely unexplored in gerontology. Some studies have been carried out to identify markers for biological aging with the assessment of physiological (based on blood pressure, respiration function etc.), biochemical (albumin, cholesterol etc.), genetic (telomere length) or epigenetic (DNA methylation) parameters. These studies suggest that individuals showing certain parameters with values similar to older individuals live shorter, thus being "biologically older" than those found in the majority of the individuals of the same chronological age (Bae *et al*., 2008; Belsky *et al*., 2015; Jylhävä *et al*., 2017; Nakamura *et al*., 2007). Increasing evidence suggests that the immune system is a good indicator of health status and longevity (De la Fuente and Miquel, 2009; Wayne *et al*., 1990). Moreover, the immune system seems to reflect the degradation of biological systems due to age-related changes (De la Fuente and Miquel, 2009). Recently, several immune function parameters were validated as markers of biological age and predictors of longevity (Martínez de Toda *et al*., 2016). This study from our research group indicates that there is an age-related decline in the immune function parameters analysed in both human blood neutrophils and lymphocytes, as well as in peritoneal macrophages and lymphocytes from mice, with the lowest

values in elderly humans (65-79 years of age) and old mice (72 weeks of age). In addition, these immune function parameters were preserved in long-lived individuals (both human centenarians and extremely long-lived mice) (Martínez de Toda *et al*., 2016).

1.1.5. Theories of aging

Biological, epidemiologic, and demographic data have generated a large number of theories in an attempt to explain the process of aging and its inevitable consequence, death (Weinert and Timiras, 2003). Given that aging is extremely complex and multifactorial, more than 300 theories have been proposed to explain this process (Medvedev, 1990). Most of these theories describe only some features of the aging process. Thus, these theories should be rather considered complementary than mutually exclusive (Weinert and Timiras, 2003). To better understanding, these theories were classified into three major groups: (1) genetic theories, (2) epigenetic theories, and (3) evolutionary theories.

1.1.5.1. Genetic theories

The genetic theories support that aging is the result of programmed changes driven by gene expression following the reproductive maturation (Weinert and Timiras, 2003). In this group are included both the Hayflick´s theory and the shortening telomeres theory. The Hayflick´s theory is basically based on the idea that the somatic cells with replicative potential possess a "mitotic clock" that fixes their maximum life span (Hayflick, 1965), whereas the shortening telomeres theory is based on the progressive loss of telomeres (the caps of noncoding DNA that protect the ends of chromosomes) with each cell division, resulting in cellular senescence and aging (Harley *et al*., 1990). Both theories can be considered to explain an effect of aging, such as replicative cellular senescence, but not its cause. Moreover, these theories can only be applied to cells capable of division, thus not considering post-mitotic cells (non-proliferating cells), such as most of neurons (Goyns, 2002).

In addition, currently, there is no evidence that specific aging genes, which have been denominated gerontogenes, could have a role in causing aging. However, some authors suggest that gerontogenes when overexpressed or mutated could have an influence on survival, longevity and rate of aging (Moskalev *et al*., 2014; Rattan, 2006). For instance, mutations in certain genes for growth and development of yeasts, nematodes, flies, and mice showed to prolong their life span (Moskalev *et al*., 2014). In addition, along with this line, "the gene cluster hypothesis of aging" proposes that the maximum longevity would be under the control of gene clusters (Barja, 2008).

1.1.5.2. Stochastic theories. The free radical and oxidation theory

Most of gerontology researchers, even those who previously supported the genetic theories, currently believe that the aging process, which occurs after reproductive maturation, is driven by events that are not guided by a genetic program but are stochastic or random (Hayflick, 2007). Among the epigenetic theories, the free radical theory of aging, proposed by Harman (1956), is probably one of the most accepted nowadays to explain how the aging process occurs. According to this theory, which was further developed by other researchers (Barja 2002; 2004; Miquel, 1998; Miquel *et al*., 1980; Pamplona and Barja, 2003), aging is the result of the accumulation of oxidative damage to macromolecules caused by reactive oxygen species (ROS). These compounds are produced mostly in the mitochondria respiratory chain as a result of the necessary use of oxygen (Barja 2002; 2004; Miquel, 1998; Miquel *et al*., 1980; Pamplona and Barja, 2003).

As a variant of the free radical theory of aging, the mitochondrial theory emphasizes the important role played by the mitochondria in the process of aging. Thus, mitochondrial components, especially mitochondrial DNA (mtDNA), are the principal targets of ROS-induced cellular damage. This oxidative damage of mitochondria impairs its function, leading to bioenergetic decline and progressive loss of somatic physiological functions (Barja 2002; 2004; 2013; De la Fuente and Miquel, 2009; Miquel, 1998; Miquel *et al*., 1980; Pamplona and Barja, 2003). This is particularly

relevant in post-mitotic cells, such as most of neurons, in which regeneration of the damaged mitochondria cannot occur (Barja, 2002; Harman, 1972; Miquel, 1998; Miquel *et al*., 1980).

In addition, the inflammaging theory, proposed by Franceschi *et al*. (2000), characterizes aging as a consequence of a chronic and sterile (occurring in the absence of infection and primarily driven by endogenous signals) low-grade inflammation. A variety of stimuli sustain inflammaging during the process of aging, including pathogens (non-self), endogenous cell debris and misplaced molecules (self) and nutrients and gut microbiota (quasi-self) (Franceschi *et al*., 2000; 2018; Franceschi and Campisi, 2014).

More recently, based on these concepts, the oxidation-inflammation theory of aging has been suggested as an attempt to explain the cause of aging. This theory supports that with age appears a chronic oxidative and inflammatory stress, since both oxidation and inflammation are two closely related processes (Vida *et al*., 2014), affecting all cells and particularly those of the regulatory systems, such as the nervous, endocrine and immune systems. Thus, these systems show oxidative damage, and consequently functional losses incompatible with an adequate preservation of homeostasis (or homeodynamics), resulting in increased morbidity and mortality (De la Fuente and Miquel, 2009). In this theory, the immune system has been suggested to play an important role in the process of aging, particularly in the rate of aging, since this system can increase or decrease the oxidative-inflammatory stress of the organism (De la Fuente and Miquel, 2009). Thus, aging and its related diseases are greatly linked to oxidative and inflammatory stress (Bauer and De la Fuente, 2016; De la Fuente and Miquel, 2009).

1.1.5.3. Evolutionary theories

The evolutionary theories of aging are an attempt to explain why the aging process occurs. These theories support that the process of aging is programmed to limit population size or accelerate the turnover of generations, therefore sustaining the adaptation of organisms to changing environments (Kirkwood and Austad, 2000). According to Weisman (1891), the aging process is

necessary for the disposal of the mortal soma in order to prevent organisms competing with their progeny for food and space. Moreover, aging would be a consequence of individuals selected by evolution as an advantage for the youth subjects of the species allowing them to reach the reproductive age in the best condition and thus preserve these species, but are disadvantage for older individuals (Trindade *et al*., 2013). Thus, selection acts before the adult age and the maintenance of the species is more relevant biologically than longevity of the individuals. In contrast, the antagonistic pleiotropy theory supports the existence of pleiotropic genes that would maximize vigour in early life, but at later ages would have harmful effects (Williams, 1957).

1.1.5.4. An integrated theory of aging: how, where and why of aging

Most theories of aging support only partial explanations of the causes and effects of aging. Moreover, great part of them does not consider all the different levels of biological organization (molecular, cellular, and physiological). Given the complexity of the aging process, a theory based on only one mechanism could not provide a satisfactory explanation of all its aspects (De la Fuente and Miquel, 2009). Therefore, an integrated theory has been proposed in order to answer key questions of the aging process, such as: (1) How aging happen? Aging is caused mainly by oxidative and inflammatory stresses in which all levels of biological organization are affected, including the whole organism. (2) Where does aging start? Aging starts in the mitochondria from fixed differentiated cells. (3) Why does aging happen? This answer could be found in the evolutionary theories in which aging follows the purpose to limit population size (Bauer and De la Fuente, 2016; De la Fuente and Miquel, 2009; Harman, 1956; Weisman, 1981).

1.2. OXIDATIVE AND INFLAMMATORY STRESSES

Reactive oxygen and nitrogen species (ROS and RNS, respectively) are generated in the organism as a result of the normal cellular metabolism. At moderate amounts, they are essential for

many physiological processes, including immune response, cellular signalling, mitogenic response, and redox regulation (Valko *et al.,* 2007). However, when the amounts of these oxidants are too high and cannot be counteracted by antioxidant defences, oxidative stress is established. Oxidative stress is deleterious to biological organisms because it damages lipids, carbohydrates, proteins and nucleic acids, resulting in the breakdown of the cellular functions. Similarly, inflammatory response is necessary to repair tissues and destroy pathogens, but it has to be limited in space and time. Thus, the imbalance between the generation of pro-inflammatory and anti-inflammatory compounds in favour of the former, which is defined as inflammatory stress, is related to aging and diseases (Bauer and De la Fuente, 2016; De la Fuente, 2018a).

1.2.1. Free radicals and oxidant compounds

Almost all-living organisms require oxygen for efficient production of energy, by the use of electron transport chains that ultimately donate electrons to molecular oxygen (O_2). Nearly 95% of the reduction of oxygen to water follows the mitochondrial enzyme cytochrome oxidase pathway, without the generation of any intermediates (Fridovich, 1978; Halliwell and Gutteridge, 1999). However, the remaining 5% of oxygen reduction goes through univalent pathway in which reactive oxygen and nitrogen species (ROS and RNS) are produced (Hammond *et al*., 1985). Examples of ROS and RNS, which are a collective term that includes both oxygen and nitric oxide radicals and certain non-radicals that are oxidizing agents and/or are easily converted into radicals (Halliwell and Gutteridge, 1999), are shown in **Table 1**.

The mostly known ROS include superoxide anion radical ($O_2\bullet^-$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH\bullet$). $O_2\bullet^-$ is primarily formed with the acceptance of one electron. This oxygen free radical may act as a reducing or an oxidizing agent. Dismutation of $O_2\bullet^-$, which can occurs spontaneously or be catalysed by superoxide dismutase (SOD), produces another reactive oxygen species, hydrogen peroxide (H_2O_2). SOD prevents the accumulation of $O_2\bullet^-$, which can damage and inactive proteins containing iron-sulfur clusters (Schieber and Chandel, 2014).

Although H_2O_2 is not considered a free radical, it can be toxic at high concentrations and it can produce free radicals. H_2O_2 is formed by the two-electron reduction of oxygen or by the dismutation of $O_2^{\bullet -}$. Catalase (CAT) and glutathione peroxidase (GPx) catalyse reactions that reduce intracellular levels of H_2O_2. Thus, these enzymes, SOD, CAT and GPx, act as antioxidant defences against ROS. The accumulation of $O_2^{\bullet -}$ and H_2O_2 and the presence of a trace metal can potentially result in the production of hydroxyl radical ($OH^{\bullet}$). The $OH^{\bullet}$ radical can react at high rates with almost every cell component, which can lead to extensive cellular damage (Hammond *et al*., 1985).

The reactive nitrogen species (RNS) include nitric-derived compounds, such as nitric oxide radical ($NO^{\bullet}$), peroxynitrite radical ($ONOO^{\bullet}$), nitrogen dioxide radical ($NO_3^{\bullet}$) and nitrous acid non-radical (HNO_2). $NO^{\bullet}$ acts as a relevant signalling molecule in several physiological processes, including the regulation of blood pressure, neurotransmission, and immune response, and when combined with $O_2^{\bullet -}$, produces $ONOO^{\bullet}$, a potent oxidizing agent that cause DNA and lipid peroxidation (De la Fuente, 2018a; Vida *et al*., 2014)

Some of these species are much less "reactive" than others, for instance, $O_2^{\bullet -}$, $NO^{\bullet}$ and H_2O_2, react directly with few molecules in organism, whereas $OH^{\bullet}$ can react with anything (Halliwell and Gutteridge, 1999).

Table 1. Examples of reactive species.

Free radicals	Non-radicals
Reactive Oxygen Species (ROS)	**Reactive Oxygen Species (ROS)**
Superoxide, $O_2^{\bullet-}$	Hydrogen peroxide, H_2O_2
Hydroxyl, $OH^{\bullet}$	Hypobromous acid, HOBr
Hydroperoxyl, $HO_2^{\bullet}$	Ozone, O_3
Peroxyl, $RO_2^{\bullet}$	Singlet oxygen, $^1\Delta_g$
Alkaxyl, $RO^{\bullet}$	Hypochlorous acid, HOCl
Reactive Nitrogen Species (RNS)	**Reactive Nitrogen Species (RNS)**
Nitric oxide, $NO^{\bullet}$	Nitrous acid, HNO_2
Nitrogen radical, $NO_3^{\bullet}$	Nitroxyl anion, NO-

ROS is a collective term that includes both oxygen radicals and certain non-radicals that are oxidizing agents and/or are easily converted into radicals. RNS is also a collective term including nitric oxide radicals as well as non-radicals. Cited in Halliwell and Gutteridge, 1999.

1.2.1.1. Sources of oxygen and nitrogen species (ROS and RNS)

ROS and RNS can be produced from either endogenous or exogenous sources. The endogenous species are generated from cellular respiration and other metabolic processes. These processes include the participation of organelles, such as mitochondria, endoplasmic reticulum, peroxisome, as well as a variety of enzymes (De la Fuente, 2018a; Mittal *et al*., 2014). In contrast, exogenous sources result from pollution, alcohol, tobacco smoke, heavy metals, transition metals, industrial solvents, pesticides, certain drugs, and radiation. After penetration into the organism by different routes, these exogenous compounds are decomposed and metabolized into ROS and RNS (Pham-Huy *et al*., 2008; Phaniendra *et al*., 2015).

Most of the endogenous ROS, about 95-98%, are formed in the mitochondrial respiratory chain (mtROS). The superoxide anion radical (O_2•-) is mainly produced non-enzymatically in the mitochondrial complexes I and III (Finkel and Holbrook, 2000). ROS can also be produced in the endoplasmic reticulum by the activity of cytochrome P450 enzymes. These are important for removing or detoxifying compounds present in the environment that had been ingested (such as medicaments) (Valko *et al*., 2007). Also, peroxisomes are another source for the generation of ROS, such as H_2O_2. For instance, the β- oxidation of fatty acids is the main metabolic process that produces H_2O_2 in the peroxisomes. This organelle also contains high amounts of catalase, which prevents the accumulation of H_2O_2 (Bhattacharyya *et al*., 2014; Phaniendra *et al*., 2015; Valko *et al*., 2007).

In addition, O_2•- can be generated enzymatically by the activity of xanthine oxidase, nitric oxide synthase (NOS), and nicotine adenine dinucleotide phosphate (NADPH) oxidases (NOXs) (Mittal *et al*., 2014). Xanthine oxidase is involved in the metabolism of purines, catalysing the oxidation of hypoxanthine to xanthine and the subsequent oxidation of xanthine to uric acid. In both steps, molecular oxygen is reduced, forming O_2•- followed by the generation of H_2O_2 (Hancock *et al*., 2001). Also, nitric oxide synthase (NOS) and its three isoforms have been shown to be susceptible to the formation of O_2•-.

Importantly, the NOX family, which compromise 7 members, acts as a major producer of ROS in mammalian cells. These enzymes convert cytoplasmic NADPH into NADP+ by liberating two electrons and one proton. The proton remains in the cytoplasm whereas the two electrons are transported through the plasma/phagosomal membrane and bind two oxygen molecules resulting in the formation of two superoxide anions in the extracellular or intraphagosomal space. The NADPH oxidase complex has five subunits, namely p47phox ("phox" stands for phagocyte oxidase), p67phox, p40phox, p22phox, and gp91phox (also termed NADPH oxidase 2 (NOX2)). This complex acts mainly in phagocytic cells (such as neutrophils and macrophages) leading to increased cellular oxygen consumption and subsequently generation of ROS. This phenomenon is commonly

referred to as the "respiratory burst" because of the burst of consumption of oxygen that happens in phagocytosis. In unstimulated cells, p47phox, p67phox, and p40phox exist in the cytosol, whereas p22phox and NOX2 are in the membrane, where they occur as a heterodimeric flavoprotein, cytochrome b558. On stimulation, p47phox becomes phosphorylated and the cytosolic subunits form a complex that translocates to the membrane, where it associates with cytochrome b558 to assemble the active oxidase, which transfers electrons from the substrate to O_2, forming $O_2^{\bullet-}$ (Paravicini and Touyz, 2008). After conversion of $O_2^{\bullet-}$ to H_2O_2 by SOD, an additional step via myeloperoxidase (MPO) and inducible nitric oxide synthase (iNOS) is believed to generate hypochlorous acid and $NO^{\bullet}$, respectively. These final radicals are considered the mains ROS responsible for bactericidal activity in the phagocytes (De la Fuente, 2018a; Dan Dunn *et al*., 2015). In addition, fibroblasts, endothelial cells, vascular smooth muscle cells, and other cells have been also described to have superoxide-producing enzymes analogous to the phagocyte NADPH oxidase complex, but in these cells, the low amounts of ROS produced may function as second messengers to influence redox-sensitive signal transduction pathways (De la Fuente, 2018a; Rada and Leto, 2008).

1.2.1.2. Molecular targets of free radicals

Deoxyribonucleic acid (DNA)

Both nuclear and mitochondrial DNAs (nucDNA and mtDNA, respectively) are constantly exposed to endogenous and exogenous sources of ROS that induce DNA lesions and DNA instability, including blockage of DNA replication and transcription as well as chromosomal rearrangements. In order to maintain genomic integrity, different DNA repair pathways have evolved in cells (Gredilla, 2011). The mtDNA is more susceptible to ROS damage than the nucDNA, because it is located closer to where ROS are generated (in the inner mitochondrial membrane) and it is organized as histone-free nucleoids (with less extensive packaging than nuclear chromatin) (Bogenhagen, 2012). The accumulation of mtDNA mutations, including point mutation

and large deletion, is thought to play an important role in the process of aging (Caro *et al*., 2010). For instance, the rate of production of mtROS and the degree of oxidative damage to mtDNA correlate negatively with maximum longevity in mammals (Barja and Herrero, 2000; Valko *et al*., 2007). In addition, mtDNA fragments may insert into nucDNA contributing to aging and related diseases (Caro *et al*., 2010), this fact could support the previously mentioned idea of where aging starts.

Ribonucleic acid (RNA)

ROS can extensively damage RNA produced in the organism. Given that RNA is less compartmentalized and less compact than nuclear DNA, RNA is more susceptible to oxidative damage than DNA. Moreover, the cytoplasmic localization of RNA is near to the mitochondria where ROS are produced (Hofer *et al*., 2005).

Lipid

The membrane lipids, particularly the phospholipids containing polyunsaturated fatty acid residues are more susceptible to ROS attack. The lipid peroxidation is elicited when ROS react with the hydrogen from a methylene group (CH_2) in a fatty acid, generating a lipid peroxyl radical (LOO•). LOO• can further propagate the peroxidation process by reacting with hydrogen atoms from other lipid molecules. In addition, the membrane lipid peroxidation results in the formation of several reactive aldehydes, such as malondialdehyde (MDA), 4-hydroxynonenal (4-HNE) and acrolein. These compounds are toxic end products of lipid peroxidation and cause extensive damage to the DNA and proteins (Barrera, 2012; Marnett, 1999; Phaniendra *et al*., 2015).

Protein

ROS can damage different amino acids present in the proteins. This damage may result in loss of protein functions and/or enzymatic activity. Among the various oxidative modifications of amino acids in proteins, carbonyl formation is an early marker of protein oxidation (Phaniendra *et al*., 2015; Reznick and Packer, 1994). Methionine residues of proteins are among the amino acids most susceptible to oxidation by ROS. This oxidation leads to methionine sulfoxide in proteins,

resulting in the loss of their biological activity. There is evidence that dietary methionine restriction by 80% could be responsible for increasing maximum longevity in mammals through the decrease of ROS production (Pamplona and Barja, 2006).

1.2.2. Antioxidant defences

Antioxidant defences are a mechanism developed by organisms against ROS-induced oxidative stress. Antioxidants can be defined as molecules that neutralize free radicals by accepting or donating electron(s) to a free radical species (R•). During this electron transfer, the radical character is transferred to the antioxidant, generating an antioxidant-derived radical. This newly formed radical is significantly less reactive, longer-lived and less dangerous than those previous radicals (Valko *et al*., 2007). Superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) include the main enzymatic antioxidant defences that provide the first line of defence against ROS (including superoxide anion and hydrogen peroxide). In contrast, other endogenous and exogenous antioxidants such as glutathione (GSH) system, ascorbic acid (vitamin C), α-tocopherol (vitamin E), and polyphenols (especially flavonoids), represent the non-enzymatic antioxidants and the second line of defence against ROS. The activity of antioxidants helps to preserve an adequate redox balance, which is important for health maintenance and disease prevention (De la Fuente *et al*., 2011; De la Fuente and Miquel, 2009; Valko *et al*., 2007).

Superoxide dismutase (SOD) is an endogenous antioxidant enzyme. This enzyme catalyses the dismutation of superoxide anion (O2•-) to hydrogen peroxide (H_2O_2) and oxygen (O_2), consequently resulting in the formation of compounds less hazardous than superoxide anion. Given that SOD is a metalloenzyme, it requires a metal cofactor for its activity. The metal ions that may be bound to SOD include: (1) Iron (Fe), Fe-SOD, which is generally found in prokaryotes and chloroplasts of some plants, (2) Manganese (Mn), Mn-SOD, present in prokaryotes and mitochondria of eukaryotes and (3) Copper (Cu) and zinc (Zn), Cu/Zn-SOD, which is found mainly

in the cytosol of eukaryotes, but also present in peroxisomes (Fridovich, 1995; Ighodaro and Akinloye, 2017).

Catalase (CAT) is an endogenous antioxidant enzyme generally found in all living tissues, being abundant in cells. This enzyme uses either Fe or Mn as a cofactor and catalyses the reduction of hydrogen peroxide (H_2O_2) to water and oxygen, thus accomplishing the detoxification process initiated by SOD. CAT is mainly found in the cytoplasm and peroxisomes, but is also located in the mitochondria (Bai and Cederbaum, 2001; Chelikani *et al*., 2004; Ighodaro and Akinloye, 2017).

Glutathione peroxidase (GPx) is an intracellular antioxidant enzyme, which is involved in reducing hydrogen peroxide (H_2O_2) to water as well as lipid peroxides to alcohols. GPxs are a family of enzymes composed by at least four forms of GPx (GPx1- GPx4). GPx-1 is a selenoprotein with a selenocysteine at the active site of the enzyme. In addition, GPx-1 is the most abundant form of GPx and it is present in almost all cells, being mostly located in the cytosol and mitochondria, but also found in the peroxisome of some cells (Handy *et al*., 2009).

The non-enzymatic antioxidant tripeptide γ-glutamylcysteine (GSH) is described as the major thiol intracellular redox buffer. This cysteine-containing tripeptide exists in its reduced (GSH) or oxidized (glutathione disulfide or GSSG) forms. GSH is the predominant form and account for > 98% of total glutathione. GSH is highly present in the cytosol, nucleus and mitochondria, and it is the major soluble antioxidant in these cell compartments. In addition, GSH is synthesized in the cytosol and subsequently it is transported to the mitochondria, in which it has a relevant role. GSH in the nucleus maintains the redox state of critical protein sulfhydryls that are necessary for DNA repair and expression. Moreover, GSH can directly detoxify free radicals or act as a substrate for glutathione peroxidase in the reduction of lipid peroxyl radicals as GSH is oxidized to GSSG. GSSG in turn is reduced back to GSH by the action of glutathione reductase (GR) utilizing NADPH and forming a redox cycle. GR is a flavoenzyme and has an important role in recycling GSH. The accumulation of GSSG as well as the ratio GSSG/GSH is a good marker of oxidative stress in the organism (Lu, 2009; Masella *et al*., 2005; Valko *et al*., 2007).

The main pathways of endogenous ROS formation and elimination by antioxidant defences are shown in **Figure 1.**

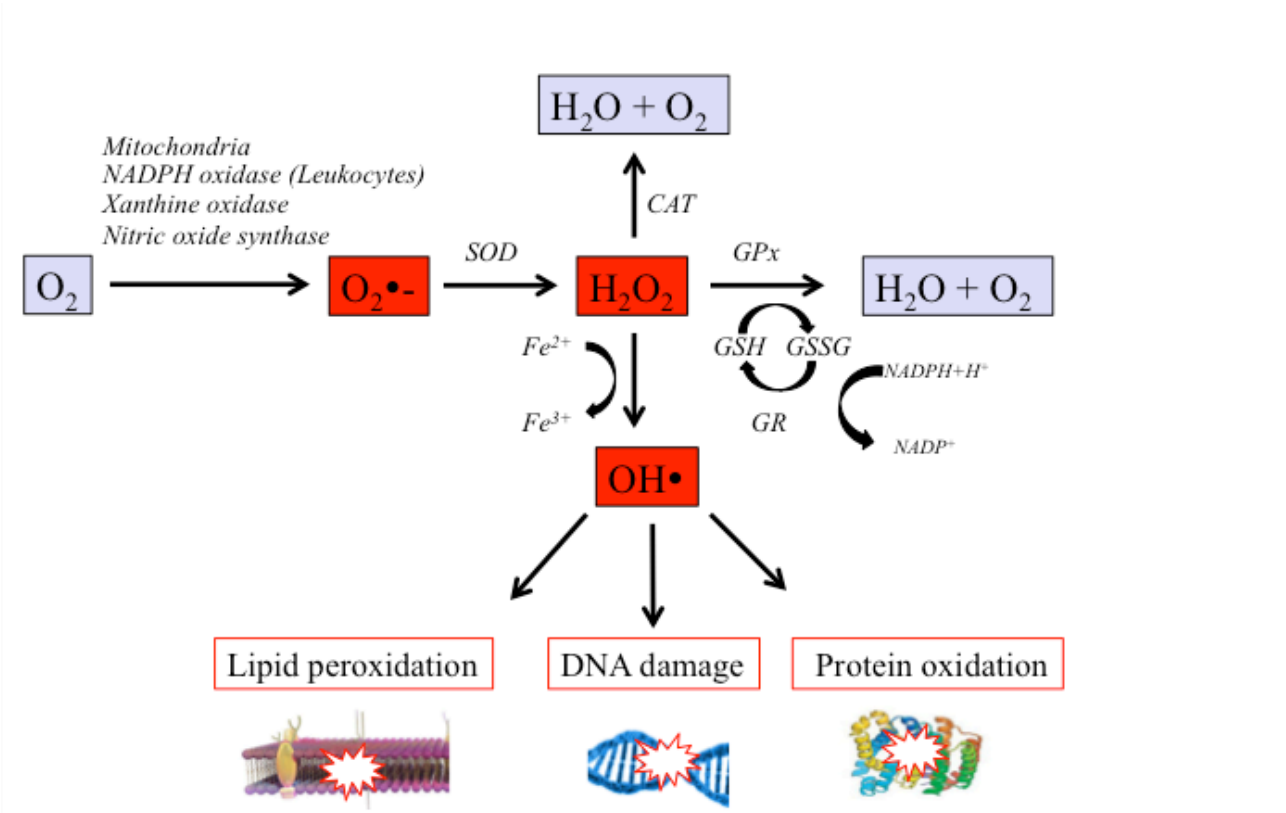


Figure 1. Main pathways of endogenous ROS generation and elimination. The superoxide anion radical (O2•-) is generated by reduction of molecular oxygen mediated non-enzymatically by redox-reactive compounds of the mitochondrial electron transport chain or enzymatically by NADPH oxidases, xanthine oxidase and nitric oxide synthase. O2•- is dismutated spontaneously or catalysed by superoxide dismutase (SOD) to produce hydrogen peroxide (H_2O_2). H_2O_2 is neutralized into water (H_2O) and molecular oxygen (O_2) by the antioxidant enzymes catalase (CAT) and glutathione peroxidase (Gpx). GPx requires reduced glutathione (GSH) as the electron donor. Oxidized glutathione (GSSG) is reduced back to GSH by the action of glutathione reductase (GR) utilizing NADPH. H_2O_2 and the presence of a trace metal (Fe^{2+}) result in the production of hydroxyl radical (OH•). The OH• radical can react at high rates with almost every cell component, with resulting lipid peroxidation, DNA damage and protein oxidation.

1.2.3. Inflammatory response

Inflammation is a beneficial response of the host against foreign challenge (such as infection) or tissue injury that has the physiological purpose of restoring tissue integrity and homeostasis (Medzhitov, 2010).

According to Medzhitov (2010), the classic inflammatory response includes four components: (1) inflammatory inducers (which initiate the inflammatory response), (2) the sensors

that detect them, such as Toll-like receptors (TLRs) and NOD (nucleotide-binding oligomerization-domain protein)-like receptors (NLRs) expressed on innate immune cells, (3) the inflammatory mediators induced by the sensors, and (4) the target tissues that are affected by the inflammatory mediators. The TLRs and NLRs induce the production of inflammatory cytokines, such as tumour necrosis factor (TNF), interleukin-1 (IL-1), interleukin-6 (IL-6) and chemokines, including chemokine (C-C motif) ligand 2 (CCL2) and C-X-X motif chemokine ligand 8 (CXCL8), as well as eicosanoids (prostaglandins, such as prostaglandin E2 (PGE2)), vasoactive amines and products of proteolytic cascades. These inflammatory mediators induce vasodilation, extravasation of neutrophils, and leakage of plasma into the target tissue. Reaching the target tissue, neutrophils attempt to kill the invading pathogens through the release of ROS. In addition, the inflammatory cytokines TNF, IL-1, and IL-6 can have systemic effects. The acute inflammatory response is generally ended by the elimination of the inflammatory inducer followed by resolution and repair phase, which is mediated by tissue-resident and recruited macrophages. Termination of the inflammatory response is a highly regulated process in which there is a switch in lipid mediators from pro-inflammatory prostaglandins to anti-inflammatory lipoxins. These latter inhibit inflammation and promote tissue repair.

Infection and tissue injury are considered drastic conditions, which are characterized by a high degree of tissue stress, and these induce a classical inflammatory response. Nevertheless, more often tissues are exposed to low levels of stressors (such as the presence of high ROS levels, hyperglycaemia or hypercholesterolemia) and as a result these tissues may malfunction. Cells of the innate immune system can sense stressed cells or tissues and mount an immune response to restore tissue homeostasis. However, the magnitude of this immune response is lower than the classic inflammatory response (Medzhitov, 2008; 2010; Xu *et al*., 2009). Medzhitov (2008) has defined this type of inflammation as para-inflammation. Para-inflammation can become chronic when stressors and tissue malfunction are present for a sustained period. Aging and several diseases, including obesity, have been highly associated with para-inflammation (also known as low-grade

inflammation) (Medzhitov, 2008). The close relationship between nutrient excess and chronic para-inflammation (or low-grade inflammation) has emerged the concept of "meta-inflammation" (Lumeng and Saltiel, 2011), meaning metabolically-triggered inflammation (Hotamisligil, 2006). In the state of meta-inflammation, M1-skewed (classically-activated macrophages) accumulate in parallel to adiposity, promoting inflammation and insulin resistance in the obese, whereas, in the lean, M2 (alternatively-activated macrophages) dominate insulin-sensitive adipose tissue (Shapiro *et al*., 2011).

In addition, the concept of "sterile inflammation", meaning the same as "para-inflammation", has also been used to describe the increased chronic levels of pro-inflammatory mediators during aging. Thus, even in the absence of infections, innate mechanisms seem to be activated, leading to a sterile inflammatory response, which if not appropriately resolved could produce chronic inflammation (Bauer and De la Fuente, 2016; Martínez de Toda *et al*., 2017).

1.3. IMMUNOSENESCENCE

A functional immune system is considered crucial for the continued survival of the organism against internal or external threats. In humans, as well as in other species, such as experimental rodents, it is well accepted that there are changes in the immune system with the aging process, a phenomenon known as immunosenescence, which leads to increased susceptibility to infectious diseases, cancer, and neurodegenerative diseases (Aw *et al.*, 2007; De la Fuente, 2008; 2018b). Immunosenescence represents a dynamic process with continuous remodelling and adaptation of the immune system components. Both innate and adaptive immune system exhibit age-related changes, which are associated with increased production of pro-inflammatory cytokines and ROS as well as lower ability to respond to new antigens. These changes are not uniform, thus some functions are enhanced (such as the pro-inflammatory activity of immune cells), whereas others are decreased or unchanged (De la Fuente and Miquel, 2009). These are further commented below.

1.3.1. Age-related changes in the haematopoietic system

Haematopoietic stem cells (HSCs) are defined by their ability to self-renewal as well as to differentiate into lymphoid and myeloid lineage cells. HSCs are localized in the bone marrow cavity, including the endosteal and vascular niches (Tamma and Ribatti, 2017). Common lymphoid progenitors (CLPs) can differentiate into T and B cells, whereas common myeloid progenitors (CMPs) can differentiate into megakaryocytes, erythrocytes, monocytes and granulocytes (neutrophils, eosinophils and basophils) (Kondo *et al*., 2003). Approximately two-thirds of the haematopoiesis activity is committed to myelopoiesis. Lymphoid and myeloid progenitors, generated by haematopoietic stem cells in the bone marrow, migrate to specialized lymphoid tissues, such as thymus (another primary lymphoid organ, in which T cells carry out their development), and several secondary lymphoid organs (spleen and lymph nodes as the most highly organized, and mucosal-associated lymphoid tissue (MALT) as less-organized lymphoid tissue, where they further mature, differentiate and acquire self/non-self discrimination (Gruver *et al.*, 2007). With age, the haematopoietic system loses its self-renewal and regenerative potential (de Haan and Lazare, 2018; Konieczny and Arranz, 2018). Moreover, there is a decline in the bone marrow cellularity, which seems to be replaced by fat adipose tissue (Compston, 2002). In addition, HSCs are skewed towards myeloid differentiation at the expense of lymphopoiesis with aging (Shaw *et al*., 2013; Konieczny and Arranz, 2018). The underlying mechanism is unclear, but probably include both aging-associated cell-intrinsic and micro-environmental changes (Shaw *et al*., 2013). In addition, dysregulation of cytokine and hormone networks associated with advanced age could affect bone marrow haematopoietic system (Compston, 2002).

1.3.2. Age-related changes in the innate immune system

Innate immunity, defined as the first line of defence against pathogens, parasites and tissue damages, has also an important role in the activation and regulation of adaptive immunity. This type

of immune defence is non-specific and acts effectively without previous exposure to pathogens. A diverse range of cell types and mechanisms mediate innate immunity, including monocytes/macrophages, natural killer (NK) and natural killer T (NKT) cells, dendritic cells (DC), granulocytes (such as neutrophils, eosinophils and basophils), as well as inflammatory mediators (such as cytokines and acute phase proteins) (Shaw *et al*., 2013). In addition, the complement system also supports innate immunity by a robust and rapid response against invading pathogens, consisting of a tightly regulated network of proteins. Complement activation results in the opsonisation of pathogens and their removal by phagocytes, as well as cell lysis. However, it should be noted that complement system also plays an important role in the adaptive immunity involving T and B cells, helping them to eliminate pathogens and to maintain immunologic memory, which prevents pathogenic reinvasion (Carroll, 2004; Sarma and Ward, 2011). Age-related alterations are observed in most of innate immune cell types. These are further commented below.

Age-related changes in phagocyte cell number

Polymorphonuclear leukocytes (PMNs), also denominated neutrophils, are the first cells to migrate to pathogen-infected tissues, thus providing the primary defence against infection. In humans, around 60-70% of blood leukocytes are granulocytes, and of these over 90% are neutrophils (Schröder and Rink, 2003). These cells are extensively generated in the bone marrow, where their production reaches up to 2 X 10^{11} neutrophils per day in a normal adult human. During infection, however, the production of neutrophils significantly increases mainly by the action of GM-CSF (granulocyte-macrophage colony-stimulating factor) (Borregaard, 2010). Also, Th17 cells-derived cytokines, such as interleukin-17 (IL-17), CXC-chemokine ligand 8 (CXCL8, also known as IL-8), interferon-γ (IFN-γ), and tumour necrosis factor (TNF), favour recruitment, activation and prolonged survival of neutrophils at inflammatory sites (Mantovani *et al*., 2011; Pelletier *et al*., 2010). Neutrophils are considered short-lived cells under *in vitro* culture conditions and thus have an estimated half-life of only 8-12h. However, *in vivo* and inflammatory-induced conditions are known to extend their survival (Mantovani *et al*., 2011). With age, the total number

of neutrophils seems to either remain stable or decrease. Thus, studies using the SENIEUR protocol (which includes healthy elderly people without comorbid conditions) showed no alteration or a mild decrease in the numbers of neutrophils (Borregaard, 2010; Chatta *et al*., 1994).

Monocytes represent about 5-10% of peripheral blood leukocytes in humans and mice. These cells, based on the differential surface expression of CD14 and Fc receptor CD16 are categorized into distinct subsets, namely classical ($CD14^{++}16^{-}$) and non-classical ($CD14^{+}16^{++}$). In addition, monocytes are precursors of tissue-resident macrophages and dendritic cells (DCs). Migration of monocytes into tissues and differentiation into macrophages or DCs is determined mostly by the inflammatory milieu (Wynn *et al*., 2013). Macrophages are resident in all tissues of the body and exhibit great anatomical functional diversity. These cells have roles in development, homeostasis, tissue repair and immunity (Wynn *et al*., 2013). As mentioned above, based on their polarization status, macrophages can be broadly categorized into classically activated or M1 macrophages or alternatively activated or M2 macrophages. Basically, M1 macrophages up-regulate pro-inflammatory mediators, whereas M2 macrophages up-regulate anti-inflammatory mediators. With age, the frequency or absolute number of monocytes generally remains unchanged, although an age-associated increase of non-classical $CD14^{+}CD16^{++}$ pro-inflammatory monocytes has been reported (Herrero *et al*., 2002; Ratts and Weng, 2012; Shaw *et al*., 2013). In addition, there are age-related changes in the balance of visceral adipose tissue macrophages toward the pro-inflammatory M1 phenotype in mice (Lumeng *et al*., 2011). In contrast, other studies showed a significant decrease in the number of peritoneal macrophages ($CD11b^{+}$) with aging (Arranz *et al*., 2010a; Puerto *et al*., 2005).

Dendritic cells (DCs) represent an important link between innate and adaptive immune responses. The plasmacytoid and the myeloid DC are antigen presenting cells, which recognize foreign antigens, take up and process them, and present the antigenic peptides in major histocompatibility complex (MHC) molecules of their surface to initiate its recognition by T cells (Steinman and Banchereau, 2007). With age, the ability of DC to prime and activate naïve CD8+ T

cells is significantly decreased, resulting in a negative impact on immune response of aged people (Della Bella *et al*., 2007; Zacca *et al*., 2015). In addition, the number of dendritic cells is unaltered (Jing *et al*., 2009) or declined with age (Della Bella *et al*., 2007).

Eosinophils, mainly involved in parasitic helminth infections and allergic diseases, can be longer lived and more biosynthetically active than usually appreciated. These cells depend on granule release for their secretory effector functions (Gordon, 2016; Rothenberg and Hogan, 2006). Eosinophil numbers have been reported unaltered with aging (Annema *et al*., 1995; Lieschke *et al*., 1989).

Age-related changes in phagocytic function

Neutrophils, monocytes, macrophages, dendritic cells, osteoclasts and eosinophils are denominated professional phagocytes (Uribe-Querol and Rosales, 2017). These cells have the function of eliminating pathogens through a process known as phagocytosis.

This process, which is shown in **Figure 2**, can be divided in several sequential stages: rolling, adherence, transmigration, chemotaxis, phagocytosis and killing of the foreign agent. In the process of phagocytosis, activated phagocytes enter post-capillary venules - adjacent to sites of bacterial infection or tissue trauma - and develop transient adhesive interaction with endothelial cells via specific classes of adhesion molecules that include integrins and selectins (Gerhardt and Ley, 2015; Zimmerman *et al*., 1992). The rolling is governed by a weak molecular interaction between the phagocytes (which express L-selectin) and endothelial cell (which express E-selectin). This interaction allows phagocytes to roll along the surface of the post-capillary venules. In contrast to rolling, which is a dynamic low-affinity adhesive interaction, adherence is a stationary high-affinity (strong) adhesive interaction between phagocytes and endothelial cells. This interaction is mediated by integrins and their ligands. Following adherence, phagocytes employ proteases to break down the endothelial monolayer and basement membrane to enter the extravascular inflammatory (exudate) site. This process is named transmigration, extravasation or diapedesis. Phagocytes also require a chemoattractant gradient in order to complete the process of migration

(Seely *et al*., 2003). Chemoattractants are soluble molecules formed by bacterial products, such as N-formyl-nethionyl-leucyl-phenylalanine or formylated peptide (fMLP) and chemokines (Cicchetti *et al*., 2002), which confer directionally on cell movement. Thus, phagocytes migrate in the direction of increasing concentration of a chemoattractant in a process named chemotaxis (Hammer and Apte, 1992; Seely *et al*., 2003). Finally, phagocytes engulf the foreign agent, resulting in the formation of a phagosome containing the antigen, which is ingested in the cytoplasm. Once the phagosome has been engulfed, lysosomes fuse with the phagosome, killing any live organism. Phagocytes - in particular, neutrophils and macrophages – are able to produce many degradative enzymes, antimicrobial peptides, and ROS for antimicrobial activity (Seely *et al*., 2003). As previously commented, the production of ROS in activated phagocyte cells (also known as the "respiratory burst") generates superoxide anion by the phagocytic NADPH oxidase (NOX-2). The oxidase is essential for microbial killing, since individuals lacking a functional oxidase suffer from enhanced susceptibility to microbial infections (De la Fuente, 2018a; Rada and Leto, 2008).

Contradictory data have been reported regarding the adherence capacity of peritoneal macrophages with aging. Thus, studies indicate both increased (De la Fuente *et al*., 2000; 2001) and unaltered adherence capacity with aging (Ortega *et al*., 2000a; 2000b). With respect to neutrophil adherence, however, most of studies indicate increased adherence capacity in non-stimulated and stimulated conditions (such as in response to formylated peptide (fMLP)) of aged individuals (Alonso-Fernández *et al*., 2008; Arranz *et al*., 2008; De la Fuente *et al*., 2008).

Following adherence, phagocyte cells migrate towards the inflammation site through a process of chemical attraction known as chemotaxis. In general, it is well accepted that there is decreased chemotactic capacity of phagocyte cells with advanced age. Thus, with age macrophages from different tissues (spleen, thymus, axillary lymph nodes and peritoneum) of rodents as well as of humans (blood neutrophils) showed decreased chemotactic capacity in response to chemoattractants, such as formylated peptides (fMLP), GM-CSF or zimosan (Alonso-Fernández *et*

al., 2008; Arranz *et al*., 2008; 2010a, De la Fuente *et al*., 2002; 2004a; 2004b; Di Lorenzo *et al*., 1999; Fulop *et al*., 2004; Niwa *et al*., 1989).

Once phagocyte cells reach the site of inflammation in the tissue, they phagocyte the foreign agent. With age, monocytes/macrophages from diverse tissues (spleen, thymus, axillary lymph nodes and peritoneum) of rodents as well as from blood neutrophils of humans showed decreased phagocytic capacity (Alonso-Fernández *et al*., 2008; Arranz *et al*., 2008; 2010a; Butcher *et al*., 2001; De la Fuente, 1985; De la Fuente *et al*., 2002; 2004a; Martínez de Toda *et al*., 2016; Sharma *et al*., 2014a; Simell *et al*., 2011).

The last stage of the phagocytic process consists in the destruction of the foreign agent. This process is carried out mainly by the production of ROS in the phagocyte cell, which has the ability of killing pathogens. With age, there is decreased generation of superoxide anion in response to stimulated conditions in peritoneal macrophages from old mice (De la Fuente *et al*., 2000; 2001) and in blood neutrophils from elderly humans (Simell *et al*., 2011; Walrand *et al*., 2006). These findings suggest a lower bactericidal capacity of phagocytic cells in elderly individuals in comparison with adult individuals. By contrast, other studies reported an increased generation of ROS in peritoneal macrophages (from old mice) and in blood neutrophils (from elderly humans) (Butcher *et al*., 2001; Ortega *et al*., 2000a; 2000b), whereas others showed this function unaltered with aging (Krause *et al*., 1999; Niwa *et al*., 1989).

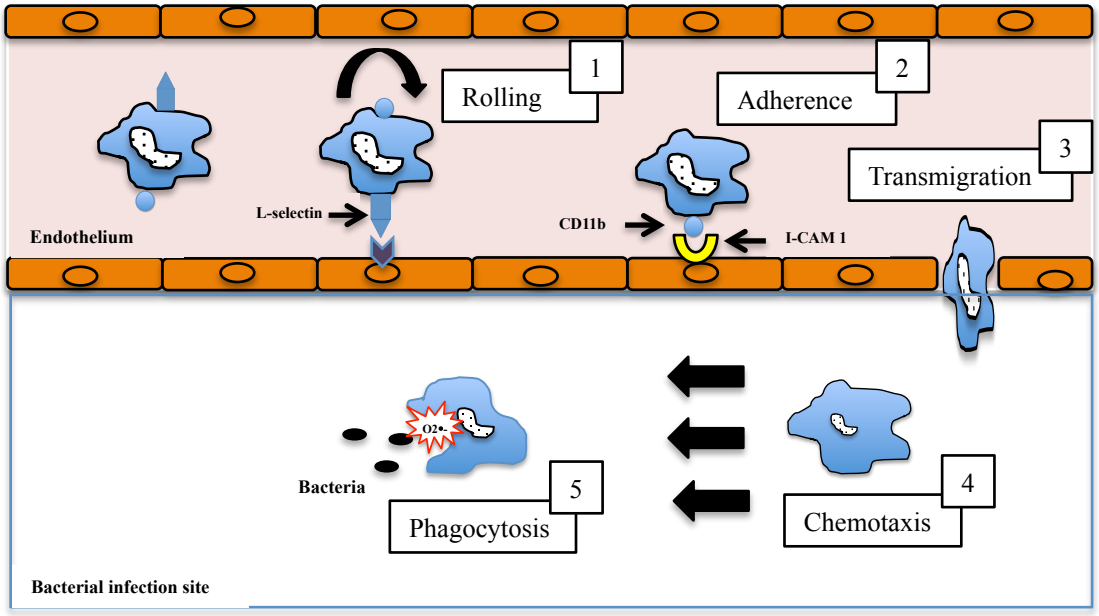


Figure 2. The process of phagocytosis by phagocytic cells. This process was divided into the sequential stages of rolling, adherence, transmigration, chemotaxis, phagocytosis and destruction of the foreign agent. ICAM-1, intercellular adhesion molecule 1.

Age-related changes in NK cell number and function

Natural killer (NK) cells are defined as effector lymphocytes of the innate immune system that participate in the early control of tumour and virus-infected cells. These cells originate from common lymphoid progenitors and mature in lymphoid tissues (spleen, bone marrow, tonsil) before entering the blood circulation, and their major difference with lymphocytes is the absence of CD3, BCR (B-cell receptor), or TCR (T-cell receptor) expression (Camous *et al.*, 2012). NK cells can mediate spontaneous cytotoxicity against abnormal cells and quickly secrete a variety of cytokines and chemokines to promote activation of adaptive immune response (Campbell and Hasegawa, 2013). NK cells are distributed throughout lymphoid and non-lymphoid tissues and compromise a minor fraction of total lymphocytes (from 2% in mouse spleen to 10% in mouse lung and from 2% to 18% in human peripheral blood) (Grégoire *et al.*, 2007). NK cells can be classified based on their CD56 surface expression. $CD56^{bright}$ cells are the immature subset, which show a high proliferative activity and the ability to release IFN-γ, TNF-α and IL-10. In contrast, $CD56^{dim}$ cells are the mature

subset, exhibiting an increased cytotoxicity activity and a decreased production of cytokines (Ponnappan and Ponnappan, 2011).

Age-related alterations are observed in the number and function of NK cells. Thus, several studies indicate increased percentage of NK cells in the peripheral blood from elderly people. In addition, there is a decrease of $CD56^{bright}$ subset and an increase of $CD56^{dim}$ subset (Solana *et al*., 1999; 2012; Solana and Mariani, 2000). However, elderly people with severe medical disorders showed decreased circulating NK cells number, as well as decreased cytotoxicity activity (Bruunsgaard *et al*., 2001). In the peritoneum of old mice, the number of NK cells was also significantly lower in comparison with adult mice (Puerto *et al*., 2005). In constrat, the number of NKT cells, a subset of T cells, was increased with aging (Solana and Mariani, 2000).

NK cells from diverse tissues (spleen, thymus, axillary lymph nodes and peritoneum) of aged mice (De la Fuente *et al*., 2002; 2004a; Ferrández *et al*., 1999; Puerto *et al*., 2005), as well as from peripheral blood of elderly humans (Arranz *et al*., 2008; 2010a; De la Fuente *et al*., 2008; Di Lorenzo *et al*., 1999; Mocchegiani and Malavolta, 2004), showed decreased cytotoxic activity. In addition, with advanced age, activated NK cells exhibited decreased production of cytokines and chemokines (Mocchegiani and Malavolta, 2004; Solana and Mariani, 2000). IL-2-induced NK proliferation was also decreased in aged individuals (Takahashi *et al*., 2003), while induction of cytotoxicity by IL-2, IL-12, or IFN-γ was maintained (Gayoso *et al*., 2011). This impaired function of NK cells in aged individuals (both rodents and humans) has been directly linked to morbidity and mortality (Aw *et al*., 2007; Bruunsgaard *et al*., 2003; Ogata *et al*., 2001).

1.3.3. Age-related changes in the adaptive immune system

The adaptive immune system, which is more recent, in evolutionary terms, than the innate immune system, is formed with diverse antigen-recognizing lymphocyte populations (naïve lymphocytes) and very long-lived antigen-experienced lymphocytes (memory lymphocytes). The former represent a specific response to any potential challenges from the universe of foreign

antigens, whereas the latter embody a rapid and robust response to subsequent encounters of a previously experienced antigen (Weng, 2006). With age, the cells responsible for maintaining immune memory overcome the cells capable of taking action (Costantini *et al*., 2018).

Age-related changes in T and B lymphocyte numbers

With age, although a reduction of naïve T cells output has been described, the overall number of T cells in the peripheral pool remains relatively stable (Bauer and De la Fuente, 2016; Hannet *et al*., 1992; Hulstaert *et al*., 1994). This could be explained possibly due to the increase of memory T cells, in both $CD4^+$ and $CD8^+$ subsets, as a result of the cumulative effects of past and persistent infections (Fagnoni *et al*., 2000; Pawelec *et al*., 2005). In addition, age-related changes are more marked in $CD8^+$ than $CD4^+$ T cells. $CD4^+$ T cells seem to maintain their number and diversity to a much older age than $CD8^+$ T cells. Thus, $CD8^+$ populations dysregulate earlier, with an increase in their number, which results in a decreased $CD4^+/CD8^+$ ratio (Bauer and De la Fuente, 2016; Boyd *et al*., 2013).

Age-related changes in B lymphocyte numbers are much less clear but appear to have some similarities to those changes in T lymphocytes. Thus, there is a decreased output of naïve B cells from bone marrow to periphery, which is associated with the concomitant increased number of long-lived memory B cells (Ongrádi and Kövesdi, 2010).

Age-related changes in T and B lymphocyte function

The most noticeable change of the aging immune system is the involution of thymus, which starts after puberty and stabilizes around 65 years old. This fact is believed to contribute significantly to immunosenescence. The thymus, called primary lymphoid organ, has an important role in the development of T lymphocytes. Thus, the expression of distinct subsets, including $CD4^+$, $CD8^+$ and NKT, occurs during T cell maturation in the thymus. These cells migrate from thymus to secondary lymphoid organs, such as the spleen and lymph nodes, where they react with foreign antigens (Kurd and Robey, 2016). The age-related thymic involution is characterized by reduction of tissue mass and thymic cellularity, loss of tissue structure and altered architecture, resulting in

the decline output of naïve T cells (Aw and Palmer, 2011). The causes of this decline seem to be associated with age-related changes in haematopoietic progenitor cells, growth factors and/or hormones, and the surrounding microenvironment of thymus (Weng, 2006). In addition, the age-related changes of T lymphocytes include the loss of expression of co-stimulatory molecules CD28, CD27, CD40L and the gain of the expression of $CD28^{+}$ cells (Costantini *et al*., 2018). Recent evidence suggests that increased frequency of CD28+CD57+ T cells were also associated with prediabetes and obesity (Lee *et al*., 2019; Yi et al., 2019).

With regards to the developmental process of B lymphocytes, it starts from naïve cells in the bone marrow (Simon *et al*., 2015). The humoral immune response of elderly individuals is characterized by decreased antibody responses (Ongrádi and Kövesdi, 2010). Antibody specificity is affected by aging, resulting in increased susceptibility to infectious diseases and decreased protective effects of vaccination (Schwab *et al*., 1989). The degree of impairment seems to be greater when T lymphocyte involvement is required to drive the antibody response (Castle, 2000). Loss of antibody diversity with age has been associated with decreased percentage and number of mature B lymphocytes. Moreover, both aged mice and humans have been associated with an increase of autoantibodies, which could predispose to the development of autoimmune diseases (Zhao *et al*., 1995).

The ability of T and B lymphocytes to adhere and migrate towards the site of inflammation, following a chemoattractant gradient, is essential for immune surveillance and recognition of antigens. These cells have the ability to recognize antigens and proliferate in response to an antigen challenge **(Figure 3)**.

Regarding the adherence capacity of lymphocytes with age, contradictory data have been reported. Thus, studies demonstrated both increased (De la Fuente and Victor, 2000; Hirokawa, 1999; McArthur, 1998) and decreased adherence capacity of lymphocytes with age (Stohlawetz *et al*., 1996).

In addition, there is a decreased chemotactic capacity of lymphocytes in diverse tissues and peripheral blood from aged mice and humans, respectively, in response to various stimuli, such as formylated peptide (fMLP), sphingosine 1-phosphate (S1P), and chemokine (C-C motif) ligand 21 (CCL21) (Arranz *et al*., 2008; De la Fuente *et al*., 2008; Huang *et al*., 2011; Martínez de Toda *et al*., 2016).

Lymphocytes are activated by antigen-specific receptors on their cell surface, which result in their proliferation and differentiation into effector cells. Thus, activated T lymphocytes can differentiate into cytotoxic T lymphocytes, whereas activated B lymphocytes can become antibody-producing cells. With age, T and B lymphocyte proliferation tend to decline, both *in vitro* (in mitogen-stimulated cultures) and *in vivo* conditions (in delayed-type hypersensitivity responses). Thus, the proliferation of lymphocytes in aged mice and humans has been described significantly decreased when exposed to mitogens specific for T lymphocytes, such as concanavalin A (ConA) and phytohaemagglutinin (PHA), and to mitogens specific for B lymphocytes, monocytes/macrophages and neutrophils, such as lipopolysaccharide (LPS) (Arranz *et al*., 2008; 2010a; De la Fuente *et al*., 2004a; 2008; Gillis *et al*., 1981; Inkeles *et al*., 1977; Martínez de Toda *et al*., 2016; 2017; Medina *et al*., 2000; Pawelec *et al*., 2002; Simioni *et al*., 2007; Song *et al*., 1993). In addition, the lymphoproliferative response to mitogens has been negatively correlated with mortality (De la Rosa *et al*., 2006; Wikby *et al*., 2005).

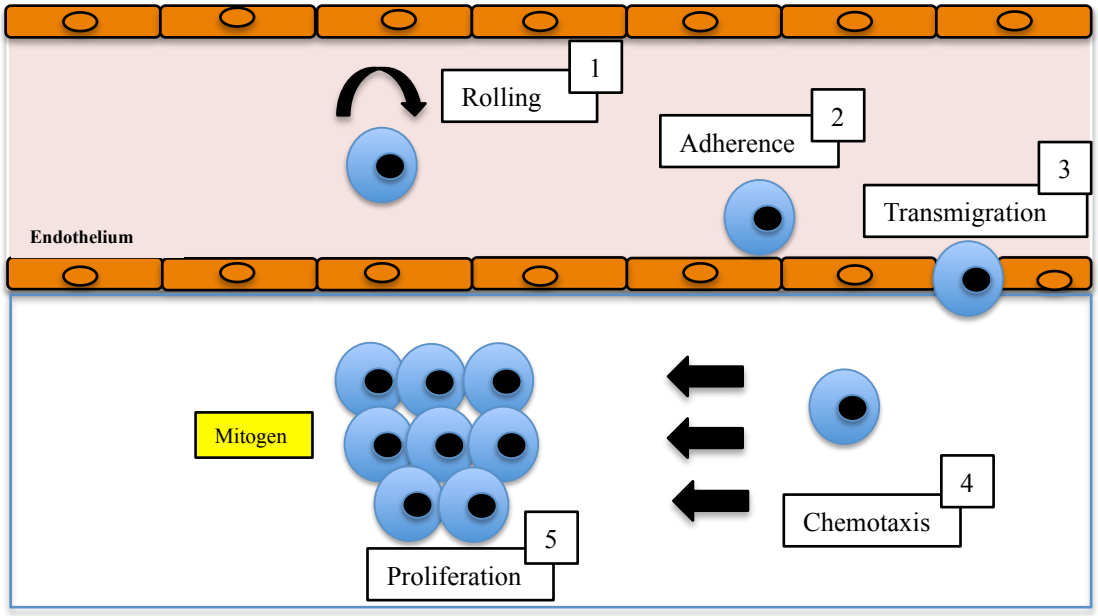


Figure 3. Lymphocyte functions. Some functions carried out by lymphocytes, including rolling, adherence, transmigration (diapedesis) and chemotaxis to the focus of infection as well as proliferation of lymphocytes in response to antigens (or mitogens, which are used in assay of laboratory).

1.3.4. Age-related changes in the cytokine levels

Cytokines, defined as small signalling proteins, mediate and regulate many biological functions, including innate and adaptive immune responses, haematopoiesis, inflammation and tissue repair. Cytokines may act on the cells that secrete them (autocrine action), on nearby cells (paracrine action), or in some instances on distant cells (endocrine or systemic action) (Zhang and An, 2007). However, with age, there is an impairment of the cytokine network in innate and adaptive immune responses (Gon *et al*., 1996; Krabbe *et al*., 2001; Lio *et al*., 1988; McNerlan *et al*., 2002). This impairment results in increased production of pro-inflammatory cytokines and of acute phase proteins (Fagiolo *et al*., 1993; Krabbe *et al*., 2001; Luciani *et al*., 2001) (a process known as inflammaging) (Franceschi *et al*., 2000). Although the production of pro-inflammatory cytokines is essential for effective immune response, such as destruction of pathogens and tumour cells, an over-production of these compounds by immune cells may lead to deleterious effects on tissues. Indeed, inflammaging is a significant risk for increased morbidity and mortality in the elderly people.

Moreover, many age-related diseases share an inflammatory pathogenesis (Franceschi and Campisi, 2014). The age-related changes in the cytokine network are further commented below.

IL-1 family

IL-1α and IL-1β are pro-inflammatory cytokines, members of the IL-1 family, and have similar biological properties (Garlanda *et al*., 2013). The IL-1α precursor is constitutively present in epithelial layers of the gastrointestinal tract, lungs, liver, kidneys, endothelial cells, and astrocytes. Upon cell death by necrosis, the IL-1α precursor is released, and functions as an "alarmin" by rapidly initiating a cascade of inflammatory cytokines and chemokines, which accounts for sterile inflammation. Thus, IL-1α mediates the early phases of sterile inflammation (Garlanda *et al*., 2013; Rider *et al*., 2011). In contrast, IL-1β is produced by haematopoietic cells, such as blood monocytes, tissue macrophages, skin dendritic cells, and brain microglia in response to toll-like receptors (TLRs), activated complement components, other cytokines (such as TNF-α), and IL-1 itself (Dinarello, 2009). The IL-1β precursor is activated by NLRP3 (nucleotide binding and oligomerization domain-like receptor family pyrin domain containing 3)/Capspase-1 inflammasome (Garlanda *et al*., 2013). Both IL-1α and IL-1β bind and activate the IL-1R that is downregulated by the receptor antagonist IL-1Rα, which blocks IL-1-mediated signal transduction. Some studies in aged people, including centenarians, reported an age-related increase in the IL-1Rα, whereas IL-1β showed no detectable age-related trend. However, studies from our laboratory showed, under basal conditions, high levels of IL-1β, and, under presence of ConA, low levels of IL-1β, in peritoneal leukocytes of old mice (Arranz *et al*., 2010c; Martínez de Toda *et al*., 2017). The age-related increase of these cytokines has been associated with high co-morbidity, age-related disease, and mortality (Cavallone *et al*., 2003; Rea *et al*., 2018; Sansoni *et al*., 2008).

IL-6

IL-6 has been recognized as an important cytokine in the process of aging and in age-related diseases. IL-6 is promptly and transiently produced in response to infections and tissue injuries, in the acute phase response, in the transition from innate to acquired immunity, in the regulation of

metabolic, regenerative, and neural processes (Weiss *et al*., 2013). It has both pro- and anti-inflammatory activities, and modulates the acute inflammatory response by producing IL-1Rα and soluble tumour necrosis factor receptor p55 (sTNF-R55), which supresses IL-1 and TNF-α (Rea *et al*., 2018). IL-6 (under basal conditions) is increased with aging and in subjects with markers of frailty and chronic disease (Arranz *et al*., 2010c; Martínez de Toda *et al*., 2017; Van Epps *et al*., 2016; Varadhan *et al*., 2014). In the presence of ConA, old mice showed decreased secretion of IL-6 (Arranz *et al*., 2010c). High basal levels of IL-6 have been correlated with cardiovascular disease, sarcopenia, muscle loss, and mortality in the elderly (Alemán *et al*., 2011; Ridker *et al*., 2000). However, in long-lived mice both stimulated and basal releases of IL-6 were very increased (Martínez de Toda *et al*., 2017). Nevertheless, in a meta-analysis of longevity in nonagenarians and centenarians, carries of the low cytokine producing IL-6 allele showed longevity benefit (Di Bona *et al*., 2009).

TNF-α

TNF-α is considered a pro-inflammatory cytokine that can be beneficial when it acts locally in the tissues (against a variety of infectious pathogens), but can have host-damaging effects when released systemically (Pfeffer, 2003). Elevated TNF-α (under basal conditions) has been reported in aged mice and humans, as well as in octogenarians with atherosclerosis, being associated with mortality (Arranz *et al*., 2010c; Bruunsgaard *et al*., 2000a; 2000b; 2003; Martínez de Toda *et al*., 2017). Elevated TNF-α concentrations has also been associated with low muscle mass and strength in old individuals (Nilsson *et al*., 1998).

IL-10

IL-10 is an important anti-inflammatory cytokine, which suppresses the actions of IL-6, TNF-α, and IL-8 (Ouyang *et al*., 2011). An age-related decline in cellular stimulation studies has been reported in peritoneal leukocytes from mice (Arranz et al., 2010c; Martínez de Toda *et al*., 2017). Although in some work the basal levels of IL-10 decrease with age (Arranz et al., 2010c; Martínez de Toda et al., 2017), in another high basal levels of this cytokine have been found in the

process of aging, probably for the purpose of neutralizing pro-inflammatory cytokines (Alvarez-Rodríguez *et al*., 2012). Interestingly, in male Sicilian centenarians, male carriers of the high producing GG 1,082 allele of the IL-10 promoter polymorphism showed a survival advantage, suggesting that IL-10 anti-inflammatory activites might be a marker for male longevity (Lio *et al*., 2003). However, this result could not be replicated in centenarians from other countries (Rea *et al*., 2018). In mice, those reaching high longevity showed an increase of IL-10 released by stimulated and basal peritoneal leukocytes (Martínez de Toda et al., 2017).

IL-2

IL-2 is a pleiotropic cytokine produced after antigen activation that plays an important role in the immune response, especially in the lymphocyte proliferation. Thus, IL-2 was first discovered as a T-cell growth factor. This cytokine promotes $CD8^+$ T cell and NK cell cytolytic activity, and modulates T cell differentiation in response to antigen, promoting naïve $CD4^+$ T cell differentiation In addition, IL-2 is essential for the development and maintenance of T regulatory cells, thus mediating tolerance and limiting inappropriate immune reactions (Liao *et al*., 2011; 2013). Many studies demonstrated that lymphocytes from aged mice and humans produce significantly lower IL-2 levels (under mitogen-induced stimulation) than adults (Arranz *et al*., 2010c; Caruso *et al*., 1996; Martínez de Toda *et al*., 2017).

Other sources of cytokines

It is important to remark that immune cells are not the only source of cytokines. For instance, adipocytes produce a variety of cytokines, known as adipokines, which have critical functions, such as modulation of metabolic pathways. However, a pro-inflammatory adipokine profile has been associated with the development of obesity and its related diseases (Deng and Scherer, 2010). Moreover, the process of aging also seems to favour an increased production of pro-inflammatory adipokines in the adipose tissue (Martyniak and Masternak, 2017).

With age, the skeletal muscle and endothelial cells have also been described as important sources of cytokines, such as IL-6 and TNF-α (Belmin *et al*., 1995; Beyer *et al*., 2012; Pedersen *et*

al., 2001). Therefore, other tissues rather than only the immune system seem to contribute to the systemic high levels of pro-inflammatory mediators, characteristic of inflammaging.

1.3.5. Oxidation-inflammation: cause and consequence of immunosenescence

As previously mentioned, the maintenance of health depends on the balance between the production of oxidants (ROS) and antioxidants. With aging, this balance is lost, due to an excess of the former and/or a lack availability of the latter, establishing an oxidative stress condition. Chronic oxidative stress has been suggested as the main contributor of immunosenescence, as well as of many age-related diseases (De la Fuente and Miquel, 2009).

The immune cells, in particular those of innate immunity, generate a great amount of ROS due to their defensive role against pathogens. For this reason, the redox maintenance in these cells is essential for its correct function. While, an insufficient amount of ROS production by immune cells would lead to a poor defensive role, an excessive production of these compounds result in their damage. In fact, immune cells are very susceptible to oxidation due to their membrane components (Meydani *et al*., 1995). In addition, with aging an oxidative stress state in the immune cells appears. Thus, decreased antioxidant capacity, including low production of reduced glutathione (GSH) concentrations, glutathione peroxidase (GPx) activity, glutathione reductase (GR) activity, superoxide dismutase (SOD) and catalase (CAT) activities, as well as a high production of oxidative compounds, such as oxidized glutathione (GSSG) concentrations, extracellular superoxide anion concentrations and xanthine oxidase (XO) activity, have been found in peritoneal immune cells from aged individuals (De la Fuente, 2008; De la Fuente *et al*. 2004b; 2005; 2008; Vida *et al*., 2014). In addition, oxidative stress has been also found in immune cells from models of premature aging, such as in prematurely aging mice (PAM) (De la Fuente, 2010; 2018a), ovariectomised mice and tyrosine hydroxylase haploinsufficient mice (Baeza *et al.,* 2011; Garrido *et al*., 2018, respectively).

Oxidative stress and inflammation are closely related, given that ROS can induce an inflammatory response. In fact, ROS themselves are considered damage-associated molecular pattern (DAMP) molecules and effectors of inflammation. Thus, DAMPs (and concretely ROS) can activate the TLR2 (toll-like receptor-2) and TL8 pathways, which initiate an inflammatory response with activation and release of pro-inflammatory cytokines. In addition, the activation of inflammasomes by DAMPs and ROS also increases the processes of oxidation and inflammation through the release of pro-inflammatory cytokines. Among the different inflammasome complexes, the pyrin domain containing 3 (NLRP3) is one of the most implicated in the release of pro-inflammatory cytokines. This NLRP3 inflammasome is directly activated by the presence of sustained amounts of ROS (De la Fuente, 2018a; Kulinsky, 2007; Vida *et al*., 2014). Although inflammation is needed for physiological functions, such as immune response, an excess of inflammatory compounds that cannot be counteracted by anti-inflammatory defence results in inflammatory stress (Kulinsky, 2007). In fact, oxidative and inflammatory stresses have been suggested as the main risk factor underlying aging and its related diseases (Bauer and De la Fuente, 2016).

1.3.6. Immune system as a marker of biological age and predictor of longevity

As previously mentioned, the preservation of immune response has been suggested as a predictor not only of health, but also of longevity (De la Fuente and Miquel, 2009; Wayne *et al*., 1990). In this sense, our research group has recently proposed parameters of immune functions, including chemotaxis and phagocytosis of macrophages, anti-tumour activity of NK cells, as well as chemotaxis and proliferative response of lymphocytes, as biological age markers. These immune function parameters, analysed in both human blood leukocytes and mouse peritoneal leukocytes, have been validated as markers of biological age and predictors of longevity (Martínez de Toda *et al*., 2016). Thus, studies performed on individuals reaching an extreme old age (human centenarians and long-lived mice), as well as those with premature immunosenesence and lower life expectancy

confirmed this concept. Interestingly, centenarians and long-lived mice exhibited a variety of immune function and oxidative stress parameters with similar values of adults (Alonso-Fernández *et al*., 2008; Arranz et al., 2010a; Bagnara *et al*., 2000; Fagiolo *et al*., 1993; Martínez de Toda *et al*., 2016; Paganelli *et al*., 1992). Therefore, parameters of immune system functions seem to be a valid method to measure the biological age of individuals, and consequently their rate of aging. Thus, adult individuals who immune function parameters show values similar to subjects of older ages would have a greater biological age and a shorter longevity, whereas those who show values similar to younger individuals would have a lower biological age and a higher longevity (Martínez de Toda *et al*., 2016; 2017).

1.4. THE NEUROIMMUNOENDOCRINE SYSTEM

The regulatory systems, including the nervous, immune and endocrine systems, are involved in the maintenance of homeostasis (or homeodynamics), and therefore of health. The functions of these systems are mutually coordinated in order to response to any change originated from the internal or external environment (Besedovsky and del Rey, 2007).

The neuroimmunoendocrine system and their communication are mainly based on the following facts: 1) neural, immune and endocrine cells can express receptors for neurotransmitters, cytokines and hormones, 2) neural, lymphoid and endocrine tissues are able to synthesize and release neurotransmitters, cytokines and hormones, and 3) functions of the nervous, immune and endocrine systems are modulated by neurotransmitters, cytokines and hormones (Besedovsky and del Rey, 1996).

Therefore, the nervous, immune and endocrine systems are intimately connected and share a bidirectional communication. Moreover, these systems do not function as independent systems. Thus, any situation capable of disrupting homeostasis (or homeodynamics) will be recognized by one of these systems and communicated to the other, in order to restore homeostasis (Wrona, 2006).

The immune system represents a system capable of detecting non-cognitive stimuli (such as viruses, bacteria, tumour cells, or other types of foreign cells) and it responds to these stimuli by producing cytokines that will be received by the neuroendocrine system. In addition, the neuroendocrine system is capable of detecting cognitive stimuli (such as light, sound, stress situations, etc.) to which it responds, and its mediators (neurotransmitters and hormones) communicate it to the immune system (Blalock, 1984).

1.4.1. Age-related changes in the neuroimmunoendocrine system

With age, the regulatory systems, including the nervous, immune and endocrine systems, as well as the communication between them, show impairments and a lower ability to adaptively react to internal and external perturbations and maintain homeostasis (or homeodynamics).

Studies from our research group support the idea that the impairment of the immune system with age can affect the functions of the other regulatory systems through increased oxidative and inflammatory stresses, resulting in increased morbidity and mortality (De la Fuente and Miquel, 2009). In fact, increasing evidence suggests that immunosenescence in mouse and human affects the central nervous system and promotes neuronal dysfunction (Deleidi *et al*., 2015). Thus, immunosenescence has shown to induce brain aging, cognitive deficit, and memory loss (Costantini *et al*., 2018). It is difficult to determine precisely whether with aging immune changes induce neural changes or an altered nervous system induces immunity changes, or whether both processes occur simultaneously, which is the most likely mechanism according to some authors (De la Fuente, 2018b; De la Fuente and Miquel, 2009).

The inadequate response to stress is one of the conditions that may lead to an acceleration of aging. Thus, studies from our research group have shown that mice with chronic hyperactivity to stress and anxiety exhibited premature immunosenescence and, consequently, premature aging (De la Fuente and Gimenez-Llort, 2010; De la Fuente 2018a; 2018b; Guayerbas *et al*., 2002a; 2002b; Guayerbas and De la Fuente, 2003).

1.5. OBESITY

1.5.1. Prevalence of obesity

The rising prevalence of overweight and obesity in several countries has been described as a global pandemic (Roth *et al*., 2004). Obesity is the fifth leading cause of death, estimated to be associated with 3.4 million deaths in 2010 (Smith and Smith, 2016). Studies forecast that the growth in obesity could lead to future falls in life expectancy (Olshansky *et al*., 2005). Increases in the prevalence of overweight and obesity have been described in men and women, in all age groups, even among children and elderly people (Samper-Ternet and Al Snih, 2012). The worldwide prevalence of overweight and obesity increased from 28.8% in 1980 to 36.9% in 2013 for men and 29.8% to 38% for women. In addition, there have been substantial increases in the prevalence among children and adolescents in developed countries, with 23.8% of boys and 22.6% of girls being either overweight or obese (Ng *et al*., 2014). In European elderly population, aged 65 years or older, the prevalence of overweight and obesity is also close to 30% (Mathus-Vliegen, 2012). Although current trends in obesity seem stable in most developed countries, morbid obesity in many of these countries continuous to rise, particularly among children (Hruby and Hu, 2015).

1.5.2. Definition and diagnosis of obesity

Obesity may be defined as an energy imbalance between calories consumed and calories expended, which results in an abnormal and excessive fat accumulation (Kopelman, 2000).

Excess of body fat (and obesity) can be assessed using different methods and tools, with variable costs, practicality and accuracy (Bacchi *et al*., 2017). For instance, dual-energy X-ray absorptiometry (Bacchi *et al*., 2017) and magnetic resonance imaging (MRI) (Abate *et al*., 1994; Andres *et al*., 2011) are considered to have high accuracy in estimating body fat mass. However, these methods are expensive and depend on sophisticated equipment, only available in some research settings (Bacchi *et al*., 2017).

Another method to estimate body fat mass is by measuring skinfold thicknesses, which is a simple and inexpensive approach. However, this method shows a high variability of fat distribution across sex, ethnicity, body type, and age (and is also technique-dependent) (Reilly *et al*., 1995). For practical reasons, the measurement of body mass index (BMI) has been considered the most used method to estimate body fat mass. BMI in adults is calculated as weight in kilograms (Kg) divided by the square of height in meters (m^2), values between 25 and 29.9 Kg/m^2 and above 30 Kg/m^2 are defined as overweight and obese, respectively. In children, BMI percentiles of historical normal groups of the same age and sex are used to diagnose obesity. Although BMI has a high correlation with body fat mass, it does not quantify total body fat adiposity or provide information about body fat distribution (Hubbard, 2000). In addition, BMI does not discriminate between fat mass and fat-free mass, being consider an inaccurate measure for very muscular individuals, such as athletes, leading to their misclassification as overweight or obese. Moreover, elderly individuals may appear to have a normal BMI despite having excess fat and reduced muscle mass (Racette *et al*., 2003).

1.5.3. Aetiology of obesity

Obesity is the result of complex interactions of multiple factors, including genetic, behavioural, environmental and physiological factors (Racette *et al*., 2003).

With regards to genetic factors causing obesity, single-gene disorders, which can be grouped in Mendelian syndrome and non-syndromic cases, account globally for less than 5% of obesity cases. Mendelian syndromes include Prader-Willi syndrome, Bardet-Biedl syndrome, Alstrom syndrome and many others. Prader-Willi syndrome (PWS) is the most common and best characterized of the human obesity syndromes. PWS is an autosomal dominant disorder characterized by obesity, mental retardation, and hyperphagia that usually develops between 12 and 18 months of age (Ochoa *et al*., 2004).

Single-gene non-syndromic disorders are known by mutations in the genes of leptin - melanocortin pathway. These may include mutations in the genes of leptin, leptin receptor, pro-

opiomelanocortin (POMC), melanocortin 4 receptor or pro-hormone convertase 1. However, the majority of obese individuals do not have a monogenic cause. Instead, these individuals generally have multiple genes that predispose them to develop obesity. Thus, polymorphisms in genes related to energy expenditure (uncoupling proteins (UCPs)) and food intake (ghrelin) have also been associated with obesity phenotypes (Ochoa *et al*., 2004).

Although genetic differences are of undoubted importance, given that individuals in the same "obesogenic" environment have different risks of becoming obese (Walley *et al*., 2009), the worldwide epidemic of obesity could be best explained by behavioural and environmental changes resulting from modern society (Kopelman, 2000; Walley *et al*., 2009).

1.5.4. Obesity-related diseases

Obesity is the major contributor to the global burden of chronic diseases. These chronic diseases include type 2 diabetes, cardiovascular diseases, respiratory diseases (such as obstructive sleep apnoea), osteoarthritis, neurodegenerative diseases, and some types of cancer. Obesity and excessive fat deposition, in particular visceral, is accompanied by profound changes in physiological functions of the organism. It is well known that hyperplasia and hypertrophy of adipocytes, as a consequence of their excessive store of energy, produce many of the health problems associated with obesity, either because of the increased weight and fat mass or because of the secretion of free fatty acids and many other pro-inflammatory adipokines from adipocytes (Bray, 2004). Stigma of obesity and the behavioural responses it produces, as well as osteoarthritis, and sleep apnoea are often associated with an increased mass of fat itself. In contrast, type 2 diabetes, insulin resistance, metabolic syndrome, hypertension, liver diseases, cardiovascular diseases, neurodegenerative diseases and some types of cancer are the result of metabolic changes associated with excessive fat (Bray, 2004).

1.5.5. Models for the study of obesity in rodents

Animal models of obesity can be classified basically into two categories: (1) animals suffering mutations or manipulations in one or a few individual genes, and (2) genetically intact animals exposed to obesogenic environments, such as high-fat diets (Lutz and Woods, 2012).

Genetic models of obesity

Studies performed in genetic models of obesity, although they are very reproducible since the animals are relatively homogenous, represent a minority percentage of the cases of obesity in the human population (De la Fuente and De Castro, 2012). Mutations in specific genes have been identified in rodents as causes of obesity. In the following are described some examples of these mutations in rodents.

Agouti gene

The agouti gene was cloned in 1993 and was the first obesity gene to be characterized at the molecular level (Lutz and Woods, 2012). The yellow mouse obesity syndrome results from a mutation at the agouti locus and is characterized by obesity, hyperinsulinemia, insulin resistance, hyperglycemia, hyperleptinemia, increased linear growth, and yellow coat colour (Moussa and Claycombe, 1999). The agouti gene codifies a protein expressed in the follicular melanocyte, where it acts as an antagonist of the melanocyte-stimulating hormone receptor (MSH), stimulates production of the yellow pigment (pheomelanin) and inhibits the pigment (eumelanin) induced by MSH. In addition, the expression of the agouti gene product, especially in the hypothalamus, antagonizes the subtype 3 and 4 melanocortine receptors (Mc3r and Mc4r), which are expressed in the hypothalamus and play an important role in the regulation of body weight (Lutz and Woods, 2012; Miltenberger *et al*., 1997).

Ob/ob syndrome

The mouse *ob/ob* syndrome was discovered in 1949 in an outbred mouse colony at the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine, and was transferred to the already well-characterized C57B1 mice colony (Zhang *et al*., 1994). *Ob/ob* mice are hyperphagic, obese,

hyperinsulinemic, and hyperglycemic, and they have been used as a model for the study of diabetes and obesity. In addition to obesity, *ob/ob* mice are infertile and have impaired immune functions. Friedman *et al*. identified in 1994 that *ob/ob* mice lacked the production of leptin (Zhang *et al*., 1994). The *ob/ob* syndrome can be reversed almost completed by exogenous leptin or transfection with the leptin gene (Lindström, 2007).

Db/db syndrome

The *db/db* mouse was identified initially in 1966 and is phenotypically similar to *ob/ob* mouse. The diabetic gene (*db*) is transmitted as an autosomal recessive trait. The major difference from the *ob/ob* mouse is the marked resistance to leptin because *db/db* mice have a (spontaneously) mutated leptin receptor. These mice also suffer from morbid obesity but their leptin levels are markedly elevated. The *db/db* mice are insulin resistant and develop diabetes (Lutz and Woods, 2012). These animals show premature immunosenescence and alterations in their behavioural responses (De Castro, 2016).

Analogous to the *db/db* mice, several rat models of leptin-resistant obesity have mutation in the leptin receptor. The obese Zucker (*fa/fa* or "fatty" rat) and the Koletsky rat carry mutated forms of the extracellular domain of the leptin receptor. This is associated with decreased leptin binding and signal transduction. They develop a similar phenotype of hyperphagia and reduced energy expenditure, leading to morbid obesity (Bray, 1977; Lutz and Woods, 2012). These rats also have impaired glucose tolerance, growth deficit, impaired immunity and infertility (De Castro, 2016; Lutz and Woods, 2012).

Diet-induced models of obesity

High-fat diets are the most common used approach to induce obesity in rodents. In general, either carbohydrate-derived calories are replaced with fat-derived calories or saturated fats (such as lard, beef tallow, coconut oil) are simply added to normal rodent chow (Bagnol *et al*., 2012). The fat content of high-fat diets, which is derived from vegetal and animal sources, can vary from 20% to 60% of total energy intake. The application of a well-controlled high-fat in which macronutrient

composition is standardized is a clear positive outcome for experimental control, enabling specific hypothesis to be generated regarding macronutrient composition and resultant phenotype (Bagnol *et al*., 2012). By contrast, some obesity researches induce obesity in rodents through the so-called cafeteria diets. In this type of diet, rodents have free access to a variety of high palatable, energy dense foods commonly consumed in western societies, and often associated with obesity. These diets are typically high in fat (>40%) and relatively low in protein. Some authors suggest that cafeteria diets are more effective to induce obesity in rodents than high-fat diets, given that the former are more palatable (Kretschmer *et al*., 2005; Sampey *et al*., 2011). However, the macronutrient composition of cafeteria diets is difficult to measure and regulate, thus conclusions regarding the role of specific nutrients to phenotype cannot be made (Bagnol *et al*., 2012).

In addition to diet, rodent species and strain are important variables to consider. In mouse, C57B16, AKR, and DBA/2 appear more prone to diet-induced obesity than other inbred strains, while SWR/J and A/J are more resistant (Prpic *et al*., 2002; Surwit *et al*., 1995). For rat strains, outbred Sprague Dawley rats are most often used (Bagnol *et al*., 2012).

1.5.6. Adipose tissue. Obesity-related changes

The adipose tissue is composed of two distinct forms, brown and white, with fundamentally different functions. The brown adipose tissue (BAT) plays an important role in body temperature regulation. In newborns, BAT is well-developed in the neck and interscapular region. Although initially thought to disappear soon after birth in humans, evidence now suggests that BAT is present in adult humans in the supraclavicular region and neck (McArdle *et al*., 2013; Yoneshiro *et al*., 2011). The brown colour of BAT is derived from a rich vascular network and abundant mitochondria and lysosomes. The mitochondria express uncoupling protein 1 (UCP-1) that is involved in the production of heat through the oxidation of the stored lipid (Frühbeck, 2008; Trayhurn, 2013). In contrast, the white adipose tissue (WAT) is integrally involved in coordinating a variety of biological processes including energy metabolism, neuroendocrine function, and

immune function (Kershaw and Flier, 2004). Thus, the classical view of the WAT as a passive reservoir for energy storage is no longer valid (Kershaw and Flier, 2004). Besides mature adipocytes, WAT contains various other cell types and is highly vascularized (Wang *et al*., 2008), and innervated (Fliers *et al*., 2003; Wang *et al*., 2008). Blood vessels and nerves connect adipose tissue to the whole body for metabolic regulation. Pre-adipocytes (immature adipocyte precursors), fibroblasts, blood cells, endothelial cells and immune cells are present in the stroma-vascular fraction (SVF) of WAT (Hauner *et al*., 1988; McArdle *et al*., 2013). In response to obesity, there is an increase in the SVF cell number (McArdle *et al*., 2013). Thus, obesity is characterized by increased number of pre-adipocytes and immune cells and these could be associated with insulin resistance (McLaughlin *et al*., 2007).

Adipocytes vary in diameter depending on the size of their single, large, lipid droplet containing triglycerides. Because around 90% of the cell volume is the lipid droplet surrounded by a film cytoplasm (containing mitochondria, endoplasmic reticulum, etc.) and bounded by a plasma membrane, the nucleus is pushed to the periphery (Frühbeck *et al*., 2001). After a meal, parasympathetic nerves stimulate adipocytes to take up fatty acids and glycerol from blood and synthesize triglycerides for storage. During fasting or when the body requires energy, sympathetic nerves acting through β-adrenergic receptors, stimulate lipolysis of adipocytes and triglycerides are hydrolysed to glycerol and free fatty acids (FFAs). FFAs, as energy substrates, are released into the circulation and are transported to the different tissues (Wang *et al*., 2008). Adipocytes are long-lived cells, with a half-life of approximately 9 years, so the excess cells can persist for a long time in obese individuals (Tchkonia *et al*., 2010). Adipocytes respond to the metabolic needs of the body and maintain the balance between synthesis (lipogenesis) and breakdown (lipolysis) of triglycerides (Wang *et al*., 2008). The regulation of these processes is responsive to hormones, cytokines, and other factors involved in energy metabolism (Frühbeck *et al*., 2001). The ability to carry out these functions is acquired during embryonic development in preparation for the postnatal period, when an adipose energy reserve becomes necessary (Cornelius *et al*., 1994). In human foetuses, the

primary fat formation is detectable between the 14th and 16th weeks of prenatal life, while the secondary fat formation takes place later in life (after the 23rd week of gestation), as well as in the early postnatal period. Adipose tissue is partitioned by connective tissue septa into lobules. The number of fat lobules remains constant, while in the subsequent developmental phases the lobules continuously increase in size. Adipogenesis, i.e. the development of adipose tissue, varies according to sex and age. Furthermore, the existence of critical periods for changes in adipose tissue cellularity has been reported. In this sense, two peaks of accelerated adipose mass enlargement have been established, namely after birth and between 9 and 13 years of age. The capacity for cell differentiation is highest during the first year of life, while it is less pronounced in the years before puberty (Bray and Bouchard, 2003). Therefore, the rate of cell proliferation slows down during adolescence and, in weight stable individuals, remains fairly constant throughout adulthood (Bray and Bouchard, 2003). In case of a maintained positive energy balance, such as obesity, adipose mass expansion takes place initially by the enlargement of existing adipocytes and subsequently by augmenting their number. Thus, the expansion of white adipose tissue is caused by a combination of size increase of preexisting adipocytes (hypertrophy) and *de novo* adipocyte differentiation (hyperplasia) (Wang *et al*., 2008). Childhood-onset obesity is characterized by a combination of fat cell hyperplasia and hypertrophy, whereas in adult-onset obesity a hypertrophic growth predominates. However, it has been shown that adult humans are capable of new adipocyte formation of cells with the ability to undergo differentiation (Frühbeck *et al*., 2001).

1.5.6.1. Adipose tissue-secreted proteins

Adipocytes secrete many hormones, growth factors, and cytokines, which have been termed adipokines. These adipokines act at both the local (autocrine/paracrine) and systemic (endocrine) level and influence several physiological processes concerning energy, glucose metabolism, and immunity (Frühbeck *et al*., 2001; Makki et al., 2013). Dysregulated expression and secretion of these adipocyte-derived proteins due to adipose tissue dysfunction can contribute to the

pathogenesis of obesity-related problems (Ouchi *et al*., 2011). Examples of adipocyte-derived proteins are described below.

Leptin is a 16-kDa polypeptide containing 167 amino acids with structural homology to cytokines, and is believed to have pro-inflammatory activities. It has been described that leptin increases the production of TNF-α and IL-6 by monocytes and stimulates the production of C-C chemokine ligands (CCL3, CCL4 and CCL5) by macrophages (Ouchi *et al*., 2011). Leptin receptors are members of the cytokine receptor class I superfamily and are expressed in both central nervous system (CNS) and periphery. Leptin is mainly secreted by adipocytes, in a direct proportion to adipose tissue mass and nutritional status, and thereby regulates food intake and energy expenditure. Caloric restriction and weight loss rapidly decline leptin levels. This decline is associated with adaptive physiological responses to starvation including increased appetite and decreased energy expenditure. In contrast, obesity is characterized by elevated circulating leptin. In addition, obesity is generally accompanied by a state of leptin resistance, which could result from defects in leptin signalling or transport across blood-brain barrier (Kershaw and Flier, 2004).

Tumour necrosis factor-alpha (TNF-α) is implicated in the pathogenesis of obesity and insulin resistance (Ruan and Lodish, 2003), given that TNF-α impairs insulin signalling (Ruan *et al*., 2002). In agreement, neutralization of TNF-induced signalling in obese animals leads to an improvement in insulin sensitivity (Uysal *et al*., 1997).

Interleukin (IL)-6 is another cytokine associated with obesity and insulin resistance (Fernández-Real and Ricart, 2003). Adipocytes and stroma-vascular cells secrete IL-6 and express its receptor (Fain *et al*., 2004), which is homologous to the leptin receptor. Thus, in contrast to TNF-α, IL-6 circulates at high levels in the bloodstream, and as much as one third of circulating IL-6 originates from adipose tissue (Fernández-Real and Ricart, 2003). In WAT, only a fraction of IL-6 is secreted by adipocytes, the other part being produced by other cells, particularly macrophages (Weisberg *et al*., 2003). IL-6 seems to decrease insulin signalling in peripheral tissues by reducing

expression of insulin receptor signalling components (Senn *et al*., 2003). IL-6 also inhibits adipogenesis and decreases adiponectin secretion (Fernández-Real and Ricart, 2003).

Macrophage and monocyte chemoattractant protein (MCP)-1 is a chemokine that recruits monocytes to sites of inflammation and it is expressed and secreted by adipose tissue, being increased in obesity (Sartipy and Loskutoff, 2003; Wellen and Hotamisligil, 2003). Both adipocytes and stroma-vascular cells have been implicated in the secretion of MCP-1. Also, MCP-1 seems to contribute to adipose tissue insulin resistance (Sartipy and Loskutoff, 2003).

Plaminogen activator inhibitor (PAI)-1 is a member of the serine protease inhibitor family and is the primary inhibitor of fibrinolysis. PAI-1 has been implicated in the angiogenesis and atherogenesis. Adipocytes and stroma- vascular cells secrete PAI-1. Plasma PAI-1 levels are elevated in obesity and insulin resistance (Mertens and Van Gaal, 2002).

Adiponectin is highly and almost exclusively synthetized in adipocytes and circulates at high levels in the bloodstream (Chandran *et al*., 2003). A strong and consistent inverse association between adiponectin and both insulin resistance and inflammatory states have been established. Adiponectin levels are low with insulin resistance due to either obesity or lipodystrophy, and administration of adiponectin improves metabolic parameters in these conditions (Chandran *et al*., 2003). In addition, adiponectin levels in the plasma and adipose tissue are decreased in obese individuals compared to lean individuals (Ryo *et al*., 2004). In agreement, the production of adiponectin by adipocytes is inhibited by pro-inflammatory cytokines, such as TNF-α and IL-6, as well as by hypoxia and oxidative stress state (Hosogai *et al*., 2007).

Adipsin is one of several adipose tissue-derived complement components that are required for the enzymatic production of acylation stimulating protein (ASP), a complement protein that affects both lipid and glucose metabolism (Cianflone *et al*., 2003). Studies indicate that both adipsin and ASP positively correlate with adiposity, insulin resistance, dyslipidemia, and cardiovascular disease (Cianflone *et al*., 2003).

Resistin is an approximately 12-kDa polypeptide that belongs to the unique family of cysteine-rich C-terminal domain proteins called resistin-like molecules. Resistin has been shown to induce resistance insulin in mice (Steppan *et al*., 2001). Resistin synthesis is restricted to adipocytes in mice (Steppan *et al*., 2001), whereas in humans is mainly produced by macrophages and monocytes, and it is not detectable in adipocytes (Savage *et al*., 2001). In human mononuclear cells, transcription of the resistin gene is induced by pro-inflammatory cytokines, including IL-1, IL-6 and TNF-α (Steppan *et al*., 2001). In addition, resistin shows pro-inflammatory properties as it promotes the expression of TNF-α and IL-6 by human mononuclear cells (Bokarewa *et al*., 2005).

Retinol-binding protein 4 (RBP4) is a hepatocyte-secreted factor that is responsible for the transport of retinol (vitamin A) throughout the body (Quadro *et al*., 1999). RBP4 is secreted by both adipocytes and macrophages. Increased serum RBP4 levels were found to associate with features of the metabolic syndrome, including high blood pressure, low levels of high-density lipoprotein, high levels of cholesterol and triglycerides, and increased body mass index. RBP4 is preferentially produced by visceral adipose tissues in states of obesity and insulin resistance, and it is a marker of intra-abdominal adipose tissue expansion and subclinical inflammation (Ouchi *et al*., 2011).

Osteopontin (OPN) acts as an extracellular matrix protein and pro-inflammatory cytokine that facilitates the recruitment of monocytes/macrophages and mediates cytokine secretion in leukocytes. In WAT from diet-induced and genetically obese mice, OPN secretion is drastically up-regulated by 40 and 80-fold, respectively (Kiefer *et al*., 2008). Interestingly, OPN deficiency not only leads to decreased adipose tissue inflammation, but also improves whole-body glucose tolerance and reduces insulin resistance in mice, independent from body composition or energy expenditure. Although OPN secretion can be induced by a variety of growth factors and cytokines, the mechanisms by which OPN is up-regulated in inflamed adipose tissue remains incompletely understood (Kahles *et al*., 2014).

Chemerin is a chemokine highly secreted in liver and WAT. Chemerin has shown to regulate adipocyte differentiation, to modulate the expression of adipocyte genes involved in

glucose and lipid homeostasis, and to enhance insulin signalling in adipocytes *in vitro* (Goralski *et al*., 2007; Takahashi *et al*., 2008). In addition, chemerin was reported to be associated with inflammation and components of the metabolic syndrome (Niklowitz *et al*., 2018).

Apelin has been described to play a role in the regulation of glucose homeostasis. High apelin serum concentrations have been found in patients with obesity, insulin resistance and liver cirrhosis. By contrast, low apelin concentrations seem to contribute to improved insulin sensitivity independently of significant weight loss (Blüher, 2014).

Vaspin is a visceral adipose-derived serine protease inhibitor that has been found significantly increased in mice with obesity and insulin resistance. It has been proposed that vaspin acts as an insulin sensitizer with anti-inflammatory effects. Several studies have assigned an important role of vaspin in the development of obesity and metabolic syndrome, but it is not clear whether it has causative or protective effect in these conditions (Dimova and Tankova, 2015).

Visfatin has an important enzymatic function in synthesizing nicotinamide mononucleotide (NMN) from nicotinamide and phosphoribosyl pyrophosphate (PRPP). Also, visfatin has been described as an adipokine predominantly secreted from visceral fat exerting insulin-mimetic effects. However, subsequent studies revealed that other tissues and adipose tissue depots may also secrete visfatin and the effects of this molecule, as an insulin mimetic, are controversial. Is has been demonstrated that hypercaloric feeding as well as aging compromise visfatin-mediated NAD+ biosynthesis and may therefore contribute to the pathogenesis of type 2 diabetes (Blüher, 2014).

Vascular endothelial growth factor (VEGF) is mostly secreted by stroma cells and by adipocytes, acting in adjacent capillaries. During adipose tissue expansion, infiltrating inflammatory cells produce a significant part of VEGF. VEGF is the main endothelial cell growth factor, being associated with the development of obesity. Animal models of obesity and obese individuals have increased VEGF concentrations, which were shown to decrease after weight loss (Letra and Seiça, 2017).

1.5.6.2. Adipose tissue immune cells

Almost all immune cell types are resident in the adipose tissue. These cells have important roles in tissue housekeeping, removal of apoptotic cells and tissue homeostasis maintenance (Schipper *et al*., 2012). However, excessive fat accumulation results in changes in the number of immune cells. Thus, it has been described that the total number of T cells, B cells, macrophages, neutrophils, and mast cells are increased in the adipose tissue of obese individuals, whereas specific subsets of T cells, including Th2, regulatory T (Treg) cell, and invariant natural killer T (iNKT) cell, as well as eosinophils are decreased (Schipper *et al*., 2012). Also, immune cell populations shift towards a pro-inflammatory profile with the production of pro-inflammatory cytokines, which both affect insulin signalling in peripheral tissues and induce β-cell dysfunction and subsequent insulin secretion defect (Klöting and Blüher, 2014). In the following are described immune cell types found in the adipose tissue.

Macrophages

Macrophages are the most abundant immune cell type found in the adipose tissue. Their number, localization, and phenotype are significantly altered during obesity. They comprise 40-60% of adipose tissue cell populations in obese mice compared to 10% to 15% of adipose tissue immune cells in lean mice (Weisberg *et al*., 2003; Xu *et al*., 2003). In addition, macrophages secrete a large amount of pro-inflammatory cytokines in response to obesity (McLaughlin *et al*., 2017). Monocytes and macrophages are extensively recruited to adipose tissue through increased expression of adipokines, including chemokines (such as MCP-1 and chemokine (C-C motif) receptor type 2 (CCR2)), and adhesion molecules, such as ICAM-1 and platelet endothelial cell adhesion (PECAM-1), among others (Osborn and Olefsky, 2012; Schipper *et al*., 2012).

In the non-obese state, macrophages predominantly exist as M2, which are induced by anti-inflammatory cytokines IL-4 and IL-13 and secrete high levels of anti-inflammatory cytokines, such as IL-10. By contrast, in the obese, the anti-inflammatory M2 population shifts towards the pro-inflammatory M1 state, which is induced by pro-inflammatory mediators, such as LPS and IFN-γ,

and secretes pro-inflammatory cytokines, including IL-6, IL-1β, iNOS, and TNF-α (McLaughlin *et al*., 2017; Schipper *et al*., 2012). Adipose tissue macrophages (ATM) cluster around necrotic adipocytes in crown-like structures (CLS). M1 macrophages decrease insulin sensitivity as a result of high TNF-α, IL-1β, and IL-6 secretion. Free fatty acids (FFAs) can activate macrophages *in vitro* acting through Toll-like receptor (TLR) 2 and TLR4. Also, FFAs induce PAI-1 secretion from macrophages *in vitro* (McArdle *et al*., 2013).

Dendritic cells (DCs)

There are not much data on the presence of DCs in the adipose tissue. The abundance of CD11c expressing inflammatory macrophages in adipose tissue probably hampers their identification because DCs also express CD11c marker (Geissmann *et al*., 2010). A study, however, showed that DCs are also present in the adipose tissue of both mice and humans and that their number are significantly increased during obesity (Bertola *et al*., 2012).

Neutrophils

The role of neutrophils in the adipose tissue inflammation has also been analysed. Plasma levels of myeloperoxidase and calprotectin (mainly secreted by neutrophils), as well as neutrophil number and activation status are higher in obese compared to non-obese individuals (Nijhuis *et al*., 2009). In mice, similar to their immediate recruitment to periphery tissues during infection, neutrophils are the earliest cell type recruited to the adipose tissue upon high-fat diet feeding. However, they are undetectable in later times points, suggesting that their recruitment is rapid and transient and precedes the recruitment of macrophages (Elgazar-Carmon *et al*., 2008).

Eosinophils

Eosinophils are found in different types of adipose tissues, especially in metabolically active perigonadal and mesenteric depots (Wu *et al*., 2011a). Studies in diet-induced (DIO) mice and genetically obese (*ob/ob*) mice showed decreased total number of eosinophils (Wu *et al*., 2011a). As a source of IL-4 in adipose tissue, they maintain the M2 macrophage state that is lost in DIO mice. Importantly, eosinophil-deficient mice have worsened glucose tolerance and insulin

sensitivity. Therefore, it appears that eosinophils have a protective role in inflammation-associated obesity by creating an anti-inflammatory environment in the adipose tissue (Wu *et al*. 2011a).

B lymphocytes

The total number of B cells in the adipose tissue is elevated in DIO mice (Winer *et al*., 2011). The finding that antibodies are preferentially located in CLSs (crown-like structures) of the adipose tissue suggests their involvement in the clearance of dead adipocytes. In addition, B cell abnormal antibody production is involved in the activation of macrophages in the adipose tissue. In addition, MHC-1 and MHC-2 molecules on B cells in this tissue show an important role in the activation of CD8+ and CD4+ T helper cells, respectively (Winer *et al*., 2011).

T lymphocytes

CD8+T cells, which are important components of adaptive immune response against viral infections, are a 3- to 4-fold increased in the adipose tissue of obese mice and humans (Winer *et al*., 2009). CD8+ T cells increase the recruitment of macrophages. Thus, depletion of CD8+ cells leads to decreased numbers of macrophages in the adipose tissue and lower levels of TNF-α and IL-6. In addition, obesity has shown to increase the number of CD4+T cells as well as Th1 cells in the adipose tissue due to antigen specific selection and expansion of T cells. In fact, Th1 cells are suggested to be one of the responsible cells for adipose tissue inflammation and insulin resistance (Nishimura *et al*., 2009).

Th17 cells are important in autoimmune diseases along with Th1 cells. Th17 specific cytokines (IL-17 and IL-23), which initiate pathogenic inflammation, were observed in the serum samples of obese individuals (Chehimi *et al*., 2017).

CD4+CD25+Foxp3+ regulatory T cells (Treg) generally play a suppressive role in inflammatory diseases and these cells seem to protect obese mice against excessive inflammation, by secreting anti-inflammatory cytokines (Feuerer *et al*., 2009). In the non-obese state, Treg cells help to maintain an anti-inflammatory environment secreting IL-10. Obesity is correlated with the decrease of Treg population (Feuerer *et al*., 2009).

Natural killer (NK) cells

NK cells play an important role in the adipose tissue. There is evidence showing increased number of NK cells in the visceral adipose tissue of diet-induced obese mice (Lee *et al*., 2016). They produce pro-inflammatory cytokines, such as TNF-α and IFN-γ, and regulate macrophages to promote insulin resistance in obesity (Wensveen *et al*., 2015). When NK cells were depleted either with neutralizing antibodies or genetic ablation in E4p4(+/-) mice, obesity-induced insulin resistance improved in parallel with decreased adipose tissue macrophage number and inflammation (Lee *et al*., 2016).

1.5.7. Obesity-related oxidative and inflammatory stresses

Obesity is often accompanied by a moderate and persistent increase of pro-inflammatory cytokines, adipokines, and other inflammatory mediators (Bondia-Pons *et al*., 2012). In addition, there is decreased production of "protective factors", such as adiponectin (Hotta *et al*., 2000). The sustained activation of immune cells as well as dysfunctional adipocytes in the adipose tissue greatly contribute to this pro-inflammatory environment of obesity, maintaining and generating a state of chronic low-grade inflammation. This pro-inflammatory environment is a potent stimulator for oxidative stress, setting a vicious cycle. The complex and close association between both increased oxidative stress and increased inflammation, not only in obesity but also in obesity-related diseases, such as type-2 diabetes, cardiovascular disease and neurodegenerative diseases, make it difficult to establish the temporal sequence of the relationship (Bondia-Pons *et al*., 2012; Cachofeiro *et al*., 2008).

When energy intake exceeds energy expenditure, the resulting state of nutrient excess can trigger stress responses in many cell types, including endothelial cells, hepatocytes, myocytes, adipocytes, and immune cells, that give rise to metabolic dysfunction. Among several adverse cellular responses to nutrient excess is the production of ROS. These compounds can be produced during oxidation of glucose and fatty acids by mitochondria. Excessive generation of ROS results in

oxidative stress, which can damage cellular structures (such as mitochondria) and trigger inflammatory response (Hotamisligil, 2006; Wisse *et al*., 2007). Excess of nutrients may also result in endoplasmic reticulum (ER) stress. ER processes newly synthesized proteins into their mature forms. ER stress gives rise to the unfold protein response, which is characterized by the accumulation of unfold protein in this organelle (de Ferranti and Mozaffarian, 2008; Wisse *et al*., 2007). ER stress and increased mitochondrial ROS can induce inflammation and oxidative stress. Another cellular response to nutrient excess is the accumulation of fatty acids derivatives that are ordinarily oxidized by mitochondria to generate ATP. The accumulation of fatty acids can result in mitochondrial dysfunction, which can create a vicious cycle by further increasing the amounts of these compounds (Lowell and Shulman, 2005; Wisse *et al*., 2007). White adipose tissue also seems to become hypoxic as adipocyte size and tissue mass expand in obesity and, this condition could contribute to the increased oxidative stress and inflammatory state associated with obesity (Trayhurn, 2013; Trayhurn and Wood, 2004). These processes share the ability to activate signalling pathways, such as jun n-terminal kinase (JNK) and the inhibitor of kappa B kinase beta (IκKβ)/nuclear factor kappa B (NFκB), that promote activation of the inflammatory response. Thus, inflammation seems to be a common endpoint (Lowell and Shulman, 2005; Wisse *et al*., 2007).

In addition, it has been suggested that the NOD-like receptor family pyrin domain containing-3 (NLRP3) inflammasome senses obesity-associated non-microbial danger signals and contributes to obesity-induced inflammation. The activation of NLRP3 inflammasome initiates downstream inflammatory cytokine production, including IL-1β and IL-18 (De Nardo and Latz, 2011; Vandanmagsar *et al*., 2011).

In addition to dysfunctional adipose tissue, increasing evidence suggests that obesity is associated with alterations in the gastrointestinal microbiota, known as dysbiosis, which can impact body fat, systemic inflammation, and insulin resistance (McLaughlin *et al*., 2017). Dysbiosis is believed to cause low-grade inflammation both systemically, through enhanced leakage of bacterial products, such as LPS, and locally in the small bowel and colon. Some of these bacterial products

are thought to accumulate and potentiate inflammation in the adipose tissue (Cani *et al*., 2007; de La Serre *et al*., 2010).

1.5.8. Obesity and the immune system

It is clear the differences between the environments in which the immune system evolved throughout most of human evolution and the current calorie-rich "obesogenic" environment (Kanneganti and Dixit, 2012). In an evolutionary context, the ability to store energy excess in the adipose tissue was essential for survival in times of food deprivation or fighting against infections. Indeed, mounting a potent immune response is energetically costly. Paradoxically, this survival characteristic is disadvantageous nowadays when food is abundant and palatable (Racette *et al*., 2003). Obesity-related changes in inflammatory status and immunity are known to contribute to the development of many chronic diseases, including type 2 diabetes, atherosclerosis, liver disease, cancer, and dementia, among others. All these diseases have an inflammatory component as a common feature (Kanneganti and Dixit, 2012; Milner and Beck, 2012).

Obesity and the immune system functions

It is well known that both malnutrition and obesity severely impair the integrity and proper regulation of the immune response (Bray, 2014). In fact, the close relationship between nutrient excess and impaired immunity (chronic low-grade inflammation) has emerged the concept of "meta-inflammation" (Lumeng and Saltiel, 2011), previously commented. Several components of the innate and adaptive immunity are impaired in obesity. These are further detailed below.

Human studies

Several studies performed in obese human individuals indicate lower proliferative response of lymphocytes T and B in response to mitogens and dysregulated cytokine expression in comparison with non-obese individuals (Lamas *et al*., 2002; Nieman *et al*., 1999; O´Rourke *et al*., 2005; Tanaka *et al*., 1993; 2001). In addition, NK cell number and cytotoxicity were significantly lower in obese subjects compared to lean controls (O´Shea *et al*., 2010). An impaired immune

response has also been observed in studies performed in obese children and adolescents. Thus, they exhibited altered lymphoproliferation in response to mitogens and decreased bactericidal capacity of polymorphonuclear leukocytes (Chandra and Kutty, 1980; Martí *et al*., 2001). In addition, obesity also impairs the proliferation of peripheral blood lymphocytes and the anti-tumour activity of NK cells in elderly individuals (Moriguchi *et al*., 1995).

Obesity has also been associated with increased morbidity and mortality from infectious diseases (Bandaru *et al*., 2013) and with poor antibody response (Huttunen and Syrjänen, 2013). Moreover, obesity is considered an independent risk factor for infection after trauma (Bochicchio *et al*., 2006; Edmonds *et al*., 2011; Serrano *et al*., 2010).

Animal studies

Different animal models of obesity have been used in many studies to analyse the effects of obesity on immune functions: genetically obese rodents characterized by mutations in the leptin gene (*ob/ob* mice), leptin receptor gene (*db/db* mice and *fa/fa* rats) and diet-induced obese rodents (Lamas *et al*., 2002).

Regarding obese (*fa/fa*) Zucker rats, many studies described impaired innate and adaptive immune responses. Thus, macrophages from these obese rats showed lower migration, phagocytosis and capacity to destroy phagocytosed yeasts compared to non-obese rats (De Castro, 2016; Plotkin *et al*., 1996). Also, obese Zucker rats exhibited lower anti-tumour NK activity in comparison with non-obese rats (De Castro, 2016; Moriguchi *et al*., 1998). In addition, these obese rats showed decreased proliferative response of splenocytes to mitogens (De Castro, 2016; Moriguchi *et al*., 1998; Tanaka *et al*., 1998). Furthermore, splenic lymphocytes from obese rats showed decreased production of IL-2, whereas increased production of pro-inflammatory cytokines (TNF-α, IL-6 and IL-1β) (De Castro, 2016; Ruth *et al*., 2008). All this could explain the greater susceptibility of genetically obese rats to infections in comparison with non-obese rats (Plotkin *et al*., 1996).

Similarly as observed in genetically obese rats, the induction of obesity by a cafeteria diet in rats also resulted in impaired immune functions. Thus, the lymphoproliferative response after LPS

or PHA stimulation was significantly impaired in diet-induced obese (DIO) rats (Lamas *et al*., 2002). Furthermore, DIO rats showed decreased oxidative burst production, suggesting a lower ability to kill bacteria in comparison with lean rats. Also, the NK cytotoxic activity against tumour cells was significantly decreased in overweight/obese rats in comparison with control rats (De la Fuente and De Castro, 2012; Lamas *et al*., 2004).

Regarding models of genetic obesity carried out in mice, particularly in *ob/ob* mice, an atrophy of lymphoid organs has been observed in these animals, affecting mainly the size and the number of cells in the thymus (Matarese, 2000). In *ob/ob* mice a decreased number of lymphocytes and NK cells, as well as an impaired NK cytotoxic activity have also been observed (Martí *et al*., 2001). In addition, phenotypic abnormalities were found in macrophages of *ob/ob* mice, such as increased production of mitochondrial superoxide and hydrogen peroxide (Lee *et al*., 1999). Another study found decreased number as well as impaired function of dendritic cells in *ob/ob* mice compared to non-obese mice (Macia *et al*., 2006). In *db/db* mice a decreased number of T lymphocytes (Kimura *et al*., 1998), an impaired chemotaxis of neutrophils (Kordonowy *et al*., 2012), an altered NK activity of splenocytes (Tian *et al*., 2002) and a decreased proliferative response of peritoneal lymphocytes to mitogens (De Castro, 2016) have also been shown. In *ob/ob* and *db/db* mice a decreased phagocytosis and capacity of killing Candida albicans have also been observed (Lindström, 2007; Loffreda *et al*., 1998; Martí *et al*., 2001). Thus, both *ob/ob* and *db/db* mice showed an increased susceptibility to bacterial infections (Milner and Beck, 2012). In particular, *ob/ob* mice displayed an increased susceptibility to Mycobacterium tuberculosis and Klebsiella pneumonia infections, whereas *db/db* mice to infections against Staphylococcus aureus and Helicobacter pylori (Bandaru *et al*., 2013).

With respect to models of obesity induced by a high-fat diet, it has been found decreased phagocytic capacity and ROS production of peritoneal macrophages (Strandberg *et al*., 2009). The number and functionality of hepatic NKT cells were also significantly lower in DIO mice in comparison with non-DIO controls (Li *et al*., 2005). An impaired function of dendritic and T cells

regarding the presentation of antigens has also been observed in DIO mice compared to non-DIO controls (Verwaerde *et al*., 2006). Furthermore, DIO mice displayed higher plasma levels of MCP-1 and number of positive CD11b macrophages/monocytes in comparison with non-DIO mice (Takahashi *et al*., 2003). DIO mice also showed decreased proliferation of spleen lymphocytes in response to ConA, PHA or LPS (Sato Mito *et al*., 2009). In addition, production of IL-2 by splenic lymphocytes from DIO mice was suppressed, whereas IFN-gamma and IL-4 production was increased (Mito *et al*., 2000).

All these studies indicate that both genetically and diet-induced obesity cause a deterioration of immune defences in rodents, affecting both the number and functionality of immune cells.

1.5.9. Obesity and the central nervous system

Increasing evidence suggests that obese individuals show an increased infiltration of oxidative and inflammatory compounds into the brain, as well as a higher generation of oxidation-inflammation in this organ in comparison with non-obese individuals. Thus, recent evidence supports the presence of neuroinflammation in the hypothalamus, amygdala, hippocampus, cortex, and cerebellum during obesity (Beilharz *et al*., 2016; Guillemot-Legris *et al*., 2016). This neuroinflammation generated in the central nervous system has been related to the development of cognitive, motor and behavioural dysfunctions (Pistell *et al*., 2010; White *et al*., 2009).

One of the earliest studies showing the effects of obesity on brain function described that brain myelination was affected in genetically obese mice (Sena *et al*., 1985). Imaging studies in humans confirmed these results and also showed myelin abnormalities associated with obesity (Jagust, 2007). Several animal studies demonstrated that diet-induced obesity was associated with cognitive alterations, such as decreased spatial learning skills (Jurdak *et al*., 2008; Molteni *et al*., 2002; Stranahan *et al*., 2008; Wu *et al*., 2003). Obesity has also been associated with anxiety, depressive-like symptoms and poor memory consolidation in rodents (Guillemot-Legris and Muccioli, 2017). Different mechanisms have been identified as being able to alter cognitive

function, including impaired levels of brain-derived neurotrophic factor, glutamatergic signalling and insulin regulation (Guillemot-Legris and Muccioli, 2017).

1.6. LEPTIN

1.6.1. Main characteristics of leptin

A search for the biological factor responsible for the homeostatic regulation of body weight was initiated by studies on animal models. In 1994, a molecular defect in the obese gene (*ob*), a gene responsible for the obese phenotype of *ob/ob* mice was identified using a positive cloning method. *Ob* gene was found on chromosome 6 in mice and on chromosome 7q31.3 in humans. The protein encoded by the *ob* gene was isolated and named leptin (from the Greek "leptos" meaning thin) (Gorska *et al*., 2010; Zhang *et al*., 1994). Leptin, a 16 kDa non-glycosylated polypeptide, is mainly produced and secreted by adipocytes in proportion to fat mass. Leptin exhibits structural similarities to members of the long-chain helical cytokine family, including leukemia inhibitory factor, ciliary neurotrophic factor, oncostatin-M and cardiotrophin-1, as well as IL-6, IL-11 and IL-12. The three-dimensional structure of the 167-amino acid leptin molecule is based on four antiparallel α-helices, connected by two long crossover links and one short loop arranged in a left-handed helical bundle, which forms a two-layer packing (Frühbeck, 2006).

Circulating leptin concentrations are correlated with the total amount of body fat, thereby reflecting the status of long-term energy stores. In addition, leptin concentrations fluctuate according to changes in caloric intake with a marked enhancement during food intake (Frühbeck *et al*., 1998; Park and Ahima, 2015). Although leptin is predominantly produced and secreted to the bloodstream by adipocytes, it is also produced at low levels by skeletal muscle, stomach, bone marrow, mammary epithelium, pituitary gland, hypothalamus, bone and placenta (Bado *et al*., 1998; Frühbeck, 2006; Hsuchou *et al*., 2009; Señarís *et al*., 1997; Wang *et al*., 1998). Initially, the effects of leptin were thought to be only centrally mediated. However, increasing evidence now suggests

that leptin has an extreme pleiotropic role. Based on its almost ubiquitous distribution of receptors, leptin has been reported to play a role in a diverse range of physiological functions both at the central nervous system and at the periphery (Park and Ahima, 2015). These functions include regulation of energy balance, brain and bone development, neuroendocrine control, reproductive function, metabolism, and immune and inflammatory functions (Allison and Myers, 2014; Otero *et al*., 2005).

In humans, most circulating leptin is bounded to serum macromolecules, which may modulate ligand bioactivity and bioavailability to target tissues (Houseknecht *et al*., 1998). In lean individuals with relatively low adipose tissue mass, the majority of leptin is in the bound form, whereas the proportion of free leptin is increased in serum of obese subjects (Frühbeck, 2006; Houseknecht *et al*., 1998). Leptin concentrations exhibit sexual dimorphism. Women tend to have higher levels than men even after body fat mass control (Frühbeck, 2006; Park and Ahima, 2015). Subcutaneous fat produces more leptin than visceral fat, and this could partly contribute to higher leptin levels in women compared to men. Leptin levels can be regulated by a variety of factors, including sex steroids, insulin, glucocorticoids, catecholamine, and cytokines (Park and Ahima, 2015).

1.6.2. Main characteristics of leptin receptor (Ob-R)

Ob-R receptor, encoded by diabetes (*db*) gene was first identified in mice using a cloning technique (Bazan, 1990). The pleiotropic nature of leptin is supported by the universal distribution of Ob-R leptin receptors. Leptin acts via transmembrane receptors and shows structural similarity to the class I cytokine receptor family (Gorska *et al*., 2010; Hegyi *et al*., 2004). Ob-R can be expressed in six alternatively spliced forms, namely Ob-Ra, Ob-Rb, Ob-Rc, Ob-Rd, Ob-Re and Ob-Rf, which have in common an extracellular domain of over 800 amino acids, a transmembrane domain of 34 amino acids and a variable intracellular domain, characteristic for each of the isoforms (Frühbeck, 2006; Lee *et al*., 1996). The isoforms of Ob-R can be classified into three categories: short, long

and secreted. The Ob-Ra isoform (the short leptin receptor isoform) appears to play an important role in transporting leptin across the blood-brain barrier (Kelesidis *et al*., 2010). The Ob-Rb isoform (the long leptin receptor isoform) mediates signal transduction and is highly expressed in the hypothalamus, an important site for the regulation of energy homeostasis and neuroendocrine function (Kelesidis *et al*., 2010). The Ob-Rb expression is also present in all types of immune cells (Gorska *et al*., 2010). The lack of the Ob-Rb isoform has been responsible for the obesity phenotypes of the *db/db* mouse and the *fa/fa* rat (Chua *et al*., 1996). Relatively weak signal transduction through long Ob-Rb isoform observed in obese and hyperleptinemic individuals is associated with delayed receptor expression on the cell surface. This fact could explain leptin resistance in these individuals (Gorska *et al*., 2010).

The binding of leptin to Ob-Rb receptor activates several signal transduction pathways, including janus kinase-signal transducer and activator of transcription-3 (JAK-STAT3), which is important for regulation of energy homeostasis and phosphatidylinositol 3-kinase (PI3K), which is important for regulation of both food intake and glucose homeostasis. Other pathways have also been described, including mitogen-activated protein kinase (MAPK), 5´adenosine monophosphate-activated protein kinase (AMPK), and the mammalian target of rapamycin (mTOR) (Kelesidis *et al*., 2010).

1.6.3. Roles of leptin in the energy homeostasis

Leptin is widely recognized for its role in regulating food intake and body mass (Anubhuti and Arora, 2008). Leptin exerts immediate effects by acting on the brain to regulate appetite. Via Ob-Rb receptor binding in the hypothalamus, leptin interacts with a complex neural circuit to control food intake, activating anorexigenic neurons (i.e. appetite-diminishing) that synthesize pro-opiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART), and inhibiting oroxigenic neurons (i.e. appetite-stimulating) that synthesize agouti-related peptide (AgRP) and neuropeptide Y (NPY). The fall in leptin stimulates the expression of AgRP and NPY

and suppresses POMC and CART, thereby increasing food intake and decreasing energy expenditure (Park and Ahima, 2015).

In addition to regulating food intake, leptin also increases energy expenditure through sympathetic nerve activity. In mice, leptin activates brown adipose tissue thermogenesis by increasing the expression of uncoupling protein (UCP)-1, but these effects have not been confirmed in humans (Kelesidis *et al*., 2010).

1.6.4. Roles of leptin in the immune system

The expression of Ob-Rb in the immune system cells (including macrophages, monocytes, neutrophils, natural killer (NK) cells, T and B cells) indicates that leptin plays an important role in the regulation of immune responses (Naylor and Petri, 2016; Zhao *et al*., 2003). In addition, leptin, which has been shown to function as a pro-inflammatory molecule (Lord *et al*., 1998), is able to modulate a variety of innate and adaptive immune system functions. These are further described below.

Roles of leptin in the innate immune system

Leptin and macrophages

Genetic leptin- and leptin receptor-deficient rodent models have been associated with impaired macrophage functions, including phagocytosis and the expression of pro-inflammatory cytokines both *in vivo* and *in vitro* (Fernández-Riejos *et al*., 2010). However, the administration of exogenous leptin was able to revert this impaired macrophage functions (Loffreda *et al*., 1998). In addition, leptin dose-dependently has shown to stimulate the production of pro-inflammatory cytokines by monocytes (such as TNF-α and IL-6) (Gabay *et al*., 2001) and to enhance the expression of CC-chemokine ligand in culture murine macrophages, through the activation of a janus kinase 2 (JAK2)-signal transducer and activator of transcription 3 (STAT-3) pathway (Kiguchi *et al*., 2009). Also, a previous study showed that, in human monocytes, leptin was able to enhance the secretion of IL-18, via activation of caspase-1 inflammasome function, acting

synergistically with ATP. This process could contribute to aberrant immune responses, such those found in type 2 diabetes and other conditions of hyperleptinemia (Jitprasertwong *et al*., 2014). Another study demonstrated that leptin stimulated the proliferation and activation of human circulating monocytes *in vitro*, promoting the expression of activation markers, such as CD69, CD25, CD38, and CD71 (Santos-Alvarez *et al*., 1999). In addition, leptin was a potent chemoattractant for mouse monocytes and macrophages, and this process required the presence of full-length leptin receptors on migrating cells (Gruen *et al*., 2007). Furthermore, leptin stimulation resulted in an increased influx of intracellular calcium in macrophages and in activated JAK/STAT, mitogen-activated protein kinase (MAPK), and phosphatidylinositol 3-kinase (PI3K) pathways, which suggests that canonical cell motility machinery was activated upon macrophage exposure to leptin (Gruen *et al*., 2007).

Leptin and dendritic cells (DC)s

Dendritic cell functions were also impaired in leptin-deficient obese mice. Thus, *ob/ob* mice have been associated with impaired number and functionality of DCs. In fact, DCs from *ob/ob* mice were less potent in stimulating allogenic T cells *in vitro* compared to those derived from wild type mice (Macia *et al*., 2006). In addition, leptin deficiency impaired the maturation of dendritic cells, which resulted in the defective production of IL-12, TNF-α, and IL-6 (Moraes-Vieira *et al*., 2014). Leptin seems to up-regulate the production of IL-1β, IL-6, IL-12, TNF-α, and MIP-1α and to down-regulate the generation of IL-10 by DCs, driving naïve T cell polarization toward a Th1 phenotype (Mattioli *et al*., 2005).

Leptin and neutrophils

Leptin has also an immunomodulatory role in neutrophils (Fernández-Riejos *et al*., 2010). Previous studies found that human neutrophils were able to express the short form of the leptin receptor, which transmited the signal inside the cell, enhancing the expression of CD11b and preventing neutrophils from apoptosis (Bruno *et al*., 2005; Procaccini *et al*., 2017; Zarkesh-Esfahani *et al*., 2004). Another study showed that leptin stimulated chemotaxis of neutrophils and

exerted by itself a chemoattractive effect comparable to that of formylated peptide (fMLP). Also, leptin was able to increase intracellular H_2O_2 production (Caldefie-Chezet *et al*., 2003). In addition, neutrophils from leptin-deficient mice exhibited impaired phagocytosis of *Klebsiella pneumoniae* and low expression of CD11b (Moore *et al*., 2003).

Leptin and Natural killer (NK) cells

Leptin has been shown to be involved in the processes of NK cell development, differentiation, activation, and cytoxicity (Tian *et al*., 2002; Zhao *et al*., 2003). Leptin seems to enhance these processes in NK cells via the activator of transcription 3 (STAT-3) and by the up-regulation of the expression of IL-2 and perforin genes (Zhao *et al*., 2003). In addition, deficient-leptin mice displayed decreased percentage and total number of NK cells in the liver, spleen, lung, and peripheral blood (Tian *et al*., 2002). Leptin seems to have a specific effect also on invariant NKT (iNKT) cells. These cells are essential for several aspects of immunity, as their dysfunction or deficiency may result in the development of autoimmune diseases. A previous study showed that Ob-R was expressed also on murine iNKT cells and that leptin suppressed iNKT cell proliferation and their cytokine production *in vitro* (Venken *et al*., 2014).

Leptin and the adaptive immune system

Leptin and T lymphocytes

Leptin has shown to play an important role in T-cell immunity. Lord *et al*. (1998) was the first to demonstrate that leptin exhibited a specific effect on T-cell responses, differentially regulating the proliferation of naïve and memory T cells. In this study, leptin specifically promoted the proliferation of naïve T cells by increasing the secretion of IL-2, through the activation of the mitogen-activated protein kinase (MAPK) and PI3-K pathway. On memory T cells, leptin favoured a pro-inflammatory phenotype response by enhancing the production of pro-inflammatory cytokines (such as IFN-γ and TNF-α) (Lord *et al*., 1998). In addition, leptin induced the expression of the adhesion molecules ICAM-1 and VLA-2 (very late antigen 2) on CD4+ T cells, possibly by the production of pro-inflammatory cytokines, such as IFN-γ (Lord *et al*., 1998). Another study showed

that the administration of human leptin (metreleptin) in women, who were affected by hypothalamic amenorrhea with acquired chronic hypoleptinemia, was able to restore both CD4+T cell counts and their proliferative response. These changes were accompanied by a transcriptional signature in which genes relevant to cell survival and hormonal response were up-regulated, and the apoptosis genes were down-regulated in circulating immune cells (Matarese *et al*., 2013). In addition, another study showed that leptin was able to promote lymphocyte survival, by up-regulating lymphocyte surface expression of glucose transporters, such as GLUT-1 and GLUT-4 (Sivitz *et al*., 1997). A previous report demonstrated that leptin seemed to be important not only in Th1-dependent immune response as described previously by Lord *et al*. (1998), but also for Th2 cell development (Batra *et al*., 2010). Leptin was also reported as a negative signal for the proliferation of human naturally occurring Foxp3+CD4CD25 regulatory T (Treg) cells. Thus, isolated human Treg cells expressed high amounts of Ob-R and produced substantial amounts of leptin that were responsible for an autocrine inhibitory loop that constrained the expansion of Treg cells (De Rosa *et al*., 2007). A recent study demonstrated decreased frequency of Th17 cells in *ob/ob* mice. Thus, obese mice with leptin or Ob-R deficiency showed decreased capacity for differentiation toward a Th17 phenotype and this condition was attributed to low activation of STAT3 and its downstream targets. These effects were restored after administration of exogenous leptin (Reis *et al*., 2015). Several studies also demonstrated that leptin was able to regulate the secretion of cytokines from human peripheral CD8+ cells. In particular, leptin enhanced the secretion of IL-2 and IFN-γ and inhibited the production of IL-4 and IL-10 (Rodríguez *et al*., 2007).

Leptin and B lymphocytes

Similar as found for T lymphocytes, leptin also plays an important role in the modulation of B lymphocytes (Procaccini *et al*., 2017). Thus, leptin deficiency has been associated with a significant decrease in lymphopoiesis, as testified by 70% fewer B lymphocytes than normal controls. Also, there were decreased counts of pre-B and immature B lymphocytes. However, treatment with recombinant leptin was able to promote an increase in B lymphocyte counts, as well

as an increase in pre-B and immature B lymphocyte counts of deficient-leptin mice (Claycombe *et al*., 2008). Similar results have also been detected in the bone marrow of fasted mice (Tanaka *et al*., 2011). In addition, another study showed that leptin stimulated the secretion of several cytokines, such as IL-6, IL-10 and TNF-α, in human B cells via activation of JAK2/STAT3 and p38 mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) 1/2 signalling pathways (Agrawal *et al*., 2011). Nevertheless, leptin seems also to induce the production of pro-inflammatory cytokines by B cells and these could play a role in the inflammation associated with chronic diseases (Gupta *et al*., 2013).

1.6.5. Roles of leptin in the neonatal development

Increasing evidence indicates that leptin has an important role in the prenatal and postnatal development. In pregnancy, the production in the placenta rises significantly in rodents and humans (Gavrilova *et al*., 1997; Señarís *et al*., 1997). In addition, there is a temporary increase of leptin levels in rodents during the early postnatal period, constituting a "leptin surge". Thus, it has been described that neonatal mice and rats experience elevated levels of leptin from postnatal day (PND) 4 to PND 14, with a peak at PND 10 (Ahima *et al*., 1998; Delahaye *et al*., 2008). However, unlike found in adult rodents, in neonates this rise in leptin is independent of fat mass or food intake (Ahima *et al*., 1998).

A great body of evidence indicates that the leptin surge is involved in the development of the central nervous system (CNS) during early life. In this sense, *ob/ob* mice showed perturbed CNS development, characterized by altered myelination and expression of neuronal and glial cell markers (Bereiter and Jeanrenaud, 1979; Sena *et al*., 1985). The administration of leptin in *ob/ob* animals during postnatal period resulted in an increase of brain size (Ahima *et al*., 1999; Steppan and Swick, 1999). However, restoration of brain weight to wild-type levels required leptin to be administered for 6 weeks, from 4 weeks of age. Administration of leptin for 2 weeks from 8 to 10 weeks of age was ineffective in the rescue of brain weight or protein content (Ahima *et al*., 1999).

Thus, it seems that there is a critical period for leptin administration in the rescue of brain development (Cottrell *et al*., 2011). In addition, the presence of circulating levels of leptin in foetal rodents and the expression of leptin receptors (Ob-Rs) throughout the CNS indicate the key role of this hormone in brain development (Hoggard *et al*., 1997; Matsuda *et al*., 1999; Udagawa *et al*., 2007).

In particular, the leptin surge has shown to contribute to the establishment of hypothalamus circuits that regulate energy balance. These seem to occur through modifications in the neuronal outgrowth and synaptic connectivity, as well as neurogenesis and neuronal and glial survival (Bouret, 2013). In addition, neurotrophic effects of leptin have also been demonstrated in extra-hypothalamic sites. Leptin administration induced ERK1/2 signalling in the cortex of both neonatal and adult mice, and in cultures embryonic cortical neurons affected growth cone size and spreading (Valerio *et al*., 2006). Taken together, these actions of leptin indicate a widespread role for this hormone in neuronal development and circuit formation (Cottrell *et al*., 2011).

1.7. UNSATURATED FATTY ACID SUPPLEMENTATION AS A NUTRITIONAL INTERVENTION TO IMPROVE IMMUNITY IN OBESITY

1.7.1. Definition and classification of dietary fatty acids

Fatty acids, both free and as part of complex lipids, play a number of key roles in metabolism, including storage and transport of energy, as essential components of cell membrane phospholipids, and as gene regulators (Rustan and Drevon, 2005). Fatty acids consist of elements, such as carbon, hydrogen, and oxygen, arranged as a linear carbon chain skeleton of variable length with a methyl group at one end (designated as omega, ω) and a carboxyl group at the other end. The letter *n* is also often used instead of the Greek ω to indicate the position of the double bond closest to the methyl group. The nomenclature for fatty acids may also indicate the location of double bonds with reference to the carboxyl group (Δ) **(Table 2)** (Rustan and Drevon, 2005). Fatty acids

can be separated into three categories: saturated, monounsaturated, and polyunsaturated. In saturated fatty acids, the carbon chain has the maximum number of hydrogen atoms attached to every carbon atom. If a pair of hydrogen atoms is missing because of a double bond between two carbon atoms, it is called an unsaturated fatty acid. A fatty acid with a single double bond is monounsaturated, whereas a fatty acid with more than one double bond is polyunsaturated (White, 2009).

The most commonly occurring saturated fatty acid (SFA) in animals, plants and microorganisms is palmitic acid (C16:0). Shorter-chain saturated fatty acids with 8-10 carbon atoms are found in milk and coconut triglycerides. SFAs have a thermodynamically more stable structure, which results in a higher melting point than unsaturated fatty acids. Thus, fats containing higher levels of saturated bonds tend to be more solid at ambient temperatures than fats containing higher levels of unsaturated bonds (White, 2009).

Most naturally occurring unsaturated fatty acids have the *cis* configuration of the double bonds. This means that the hydrogen atoms on either side of the double bond are oriented in the same direction. *Trans* isomers may be produced during industrial processing (hydrogenation) of unsaturated fatty acids and in the gastrointestinal tract of ruminants. The *cis* configuration gives a kink in the molecular shape and *cis* fatty acids have lower melting points than the *trans* fatty acids or their saturated counterparts (Rustan and Drevon, 2005). The most commonly *cis*-monounsaturated fatty acid (MUFA), found in plants and animals, is the oleic acid (C18:1 *n*-9).

The most important polyunsaturated fatty acids (PUFA) for human nutrition are those from *n*-3 and *n*-6 families. Both *n*-3 and *n*-6 PUFAs are essential nutrients, and therefore, must be obtained from the diet. Linoleic acid (C18:2 *n*-6) is a major fatty acid found in plant lipids. In animals it is derived mainly from dietary plant oils. α-linolenic (C18:3 *n*-3) is found mainly in soybean and rape seed oils and algae (Rustan and Drevon, 2005). Both essential fatty acids can be further elongated and desaturated in animal cells forming the *n*-6 and *n*-3 families of PUFA. The metabolism of the *n*-6 and *n*-3 fatty acids is competitive, since both pathways employ the same set

of enzymes. The major end product of the *n*-6 pathway is arachidonic acid (C20:4 *n*-6), which is a component of membrane phospholipids and is also a precursor of some prostaglandins, thromboxanes, and leukotrienes. It is present in animal fats and at low levels in many fish oils. This pathway is quantitatively the most important pathway of PUFA metabolism in human, given that linoleic acid is abundant in vegetable oils and vegetable oil-based products, and is therefore consumed in greater amounts than α-linolenic. The major long chain of the *n*-3 pathway are eicosapentaenoic acid (EPA, C20:5 *n*-3) and docosahexaenoic acid (DHA, C22:6 *n*-3), which are primarily found in fish oils and marine algae (Yaqoob, 2003).

Table 2. Structure of different fatty acids with a methyl end and a carboxyl (acidic) end.

ω-characteristics	Methyl end	Carboxyl end	Saturation	Δ-characteristics
Stearic 18:0		COOH	Saturate	18:0
Oleic 18:1, ω-9	9 9	COOH	Monoene	18:1 Δ9
Linoleic 18:2, ω-6	6 9	COOH	Polyene	18:2 Δ9,12
α-Linolenic 18:3, ω-3	3 15 12 9	COOH	Polyene	18:3 Δ9,12,15
EPA 20:5, ω-3	3 17 14 11 5 8	COOH	Polyene	20:5 Δ5,8,11,14,17
DHA 22:6, ω-3	3 19 16 13 10 7 4	COOH	Polyene	20:6 Δ4,7,10,13,16,19

Stearic acid is a saturated fatty acid with 18 carbon atoms and no double bond (C18:0). Oleic acid has 18 carbon atoms and one double bond in the *n*-9 position (C18:1 *n*-9), whereas eicosapentaenoic acid (EPA), with multiple double bonds, is represented as C20:5 *n*-3. It is also possible to describe fatty acids systematically in relation to the carboxyl end of the fatty acids; symbolized Δ (Greek delta) and numbered 1. All unsaturated fatty acids are shown with *cis* configuration of the double bonds. DHA, docosahexaenoic acid. Cited in Rustan and Drevon, 2005.

1.7.2. Roles of dietary fatty acids in obesity-related diseases

Epidemiological data support an association between dietary fat intake and the development of obesity. Cross-cultural studies have shown a rising body mass index in countries with high

intakes of dietary fat (Bray and Popkin, 1998). In particular, the consumption of saturated fatty acids has been linked to an increased risk of obesity and cardiovascular diseases (CVD). This effect on CVD appears to be mediated mainly by the rise of low-density lipoprotein (LDL) cholesterol concentrations (Artaud-Wild *et al*., 1993; Hu *et al*., 1997; Kagan *et al*., 1974; Keys *et al*., 1986; Mensink and Katan, 1992; Pietinen *et al*., 1997). Currently, most of dietary recommendations for CVD prevention and treatment are focused on the reduction of saturated fat intake. In general, clinical trials that have replaced saturated fat with unsaturated fat, especially PUFA, have shown a reduction in CVD events as a result of lowering both LDL and HDL (high-density lipoproteins) cholesterol concentrations. In contrast, the replacement with a high carbohydrate intake, particularly refined carbohydrate, seems to exacerbate the atherogenic dyslipidemia associated with insulin resistance and obesity that include increased triglycerides, small LDL particles, and reduced HDL cholesterol (Siri-Tarino *et al*., 2010).

However, in contrast to the detrimental effects of saturated fatty acids, MUFA and PUFA intakes have been associated with positive outcomes on obesity and obesity related-diseases. Thus, evidence from human clinical trials demonstrated that compared to carbohydrate rich diets, insulin resistant subjects consuming a diet rich in MUFA showed significantly increased fat oxidation rates and decreased abdomen-to-leg adipose ratios, thus preventing central body fat distribution (Paniagua *et al*., 2007). Similarly, the substitution of a saturated fatty acid rich diet for MUFA-rich diet for four weeks resulted in a decrease in body mass and fat mass in overweight and obese men (Piers *et al*., 2003). In addition, favourable modifications in body weight composition and amelioration of weight gain after consumption of MUFA compared to SFA have also been observed in healthy subjects (Kien, 2009). Thus, dietary MUFA consumption seems to be associated with both decreased body weight and central body fat adiposity, thus ameliorating obesity and obesity-related diseases risk (Gillingham *et al*., 2011). In addition, MUFA intake seems to prevent CVD, through the reduction of LDL-cholesterol. Thus, a meta-analysis of cohort studies reported a significant correlation between MUFA intake and decreased relative risk for CVD (Mente *et al*.,

2009). However, some studies found inconclusive data. For instance, Skeaff and Miller (2009) did not observe any effect of MUFA-rich diets on relative risk for CVD. In addition, a recent meta-analysis, which included nine cohort studies, found no significant associations between MUFA intake, circulating MUFA and risk of CVD (Chowdhury *et al*., 2014). One possible justification for these inconclusive data could be the different sources of MUFA that were used. In the case of adopting a western diet, MUFA is predominantly supplied by foods of animal origin, while in south European countries, extra virgin olive oil is the most dominant source used (Schiwingshackl and Hoffmann, 2014). Thus, studies that have used olive oil as the main source of MUFA when compared to a low-fat diet, such as the PREDIMED trial, have shown CVD benefits (Estruch *et al*., 2013). In addition, a recent meta-analysis that considered olive oil intake demonstrated an overall risk reduction of all cause mortality and of CVD mortality and events (Schiwingshackl and Hoffmann, 2014).

With regards of the effects of *n*-3 PUFA on obesity and body weight, studies have shown controversial data. Thus, Couet *et al*. (1997) demonstrated that in healthy adults the replacement of 6 g of visible fat in diet with 6 g of fish oil did not promote body weight loss. However, the interventional group exhibited lower body fat mass and increased lipid oxidation as compared to the control group. In addition, Parra *et al*. (2008) found that satiety is increased after consumption of the *n*-3 PUFAs-enriched meals. However, a recent meta-analysis performed to investigate the influence of fish oil on body composition did not find an anti-obesity benefit of *n*-3 PUFAs in overweight/obese adults. However, in this study, the waist hip ratio was improved after supplementation with fish oil in obese individuals (Du *et al*., 2015). Further larger clinical trials are needed in order to be able to recommend the most effective dosages and formulas (type of n-3 PUFA, EPA/DHA ratio) for prevention/treatment of obesity and obesity-related diseases (Lorente-Cebrián *et al*., 2013). Regarding the effects of n-3 PUFA intake on CVD, several organizations recommend increasing the consumption of EPA and DHA (at least two servings of fish per week) for primary prevention of coronary heart disease in general population (Lorente-Cebrián *et al*.,

2013). Observational studies in Inuit and Japanese populations were the first suggesting the potential cardiovascular protective role of *n*-3 PUFA, probably due to the high intake of fatty fish in their habitual diet (Bang *et al*., 1976). Later on, several controlled trials provided evidence that the consumption of *n*-3 PUFA was responsible for the protective effects of fish oils against CVD (Bang *et al*., 1980; Dyerberg and Bang, 1979). However, there is still limited evidence about the association of *n*-3 PUFAs intake and prevention in mortality from CVD. For people with specific diseases, such as hypertriglyceridemia and hypertension, the effective doses of EPA and DHA required are reported to be much higher (Lorente-Cebrián *et al*., 2013).

With regards to the consumption of *n*-6 PUFA, studies consistently have shown that replacing SFA or carbohydrate with linoleic acid decreased LDL cholesterol concentrations and total cholesterol to HDL ratio (Kris-Etherton and Yu, 1997; Mensink *et al*., 2003). In addition, a meta-analysis based on 13 cohort studies showed that high linoleic acid intake was associated with a 15% lower risk of CVD events (Farvid *et al*., 2014).

1.7.3. Roles of dietary fatty acids in the immune system

It is largely known that fatty acids can influence the immune system. Thus, extensive research over the last years suggests that the quality of dietary fat intake has an important role in modulating immune and inflammatory responses. According to Calder (2008), fatty acids have a variety of roles in immune cells, which include (1) fuels for generation of energy, (2) components of cell membrane phospholipids contributing to their physical and functional properties, (3) covalent modifiers of protein structure influencing the cellular location and function of these molecules, (4) regulators of gene expression either through effects on receptor activity, on intracellular signalling processes, or on transcription factor activation, and (5) precursors for synthesis of bioactive lipid mediators like prostaglandins, leukotrienes, lipoxins and resolvins.

It is well accepted that the influence of fatty acids on immune and inflammatory cell responses involves their incorporation into cell membrane phospholipids (Calder, 2008). For

instance, the fatty acid composition of immune cells from rodents, maintained on normal laboratory chow typically, contained 15-20% of fatty acids as arachidonic acid and very low amounts of EPA and DHA (Calder *et al*., 1990; 1994). However, changes in the dietary fatty acid intake of these animals were able to alter the fatty acid composition of immune cells (Kew *et al*., 2003; Peterson *et al*., 1998; Wallace *et al*., 2000; 2001; Yaqoob *et al*., 1995). Thus, inclusion of arachidonic acid or EPA and DHA in the diets fed to rodents resulted in high amounts of these fatty acids in the immune cells (Kew *et al*., 2003; Perterson *et al*., 1998; Wallace *et al*., 2000; 2001; Yaqoob *et al*., 1995). Similarly, studies in human immune cells also confirmed these results. Thus, an increase of arachidonic intake resulted in high amounts of this fatty acid in mononuclear cells (MNCs) (Thies *et al*., 2001), while an increase of α-linolenic acid intake resulted in high amounts of this fatty acid in MNCs and neutrophils (Caughey *et al*., 1996; Healy *et al*., 2000). In both animal and human experiments, the incorporation of *n*-3 fatty acids into membrane phospholipids of immune cells occurred in a time and dose-dependent fashion and was largely at the expense of *n*-6 PUFA, especially arachidonic acid (Browning *et al*., 2012; Healy *et al*., 2000; Rees *et al*., 2006; Yaqoob *et al*., 2000).

Roles of saturated fatty acids in the immune system

Saturated fatty acids seem to stimulate adipose tissue inflammation, which is highly associated with obesity, by a process that involves Toll-like receptor 4 (TLR4), a receptor that binds bacterial lipopolysaccharide (LPS). TLR4 activation by saturated fatty acid has shown to increase the expression of a number of inflammatory genes in adipocytes by a NFκB-dependent mechanism, similar to TLR4 activation by LPS. An excess of saturated fatty acids can also stimulate inflammatory molecules in macrophages (Chait and Kim, 2010).

Regarding the effects of high-saturated-fat diet on animal models, contradictory data have been found. Thus, some studies showed that high-saturated-fat diet did not affect lymphocyte proliferation, cytokine production or NK cell activity compared to low-fat diets (Alexander and Smythe, 1988; Locniskar *et al*., 1983; Yaqoob and Calder, 1995a; 1995b), whereas others

demonstrated a suppressive effect of this type of diet (Friend *et al*., 1980; Morrow *et al*., 1985; Ossman *et al*., 1980; Sanderson *et al*., 1995; Yaqoob *et al*., 1994a; 1994b).

Roles of monounsaturated fatty acids in the immune system

Several studies support that olive oil, rich in oleic acid, is capable of modulating functions of the immune system cells. For instance, a study with animals reported a significant inhibitory effect of olive oil on the proliferation of mesenteric lymph node lymphocytes in response to the T-cell mitogen, ConA, when compared to animals fed low-fat diet or high-fat diet (rich in hydrogenated coconut oil or safflower oil) (Yaqoob *et al*., 1994a). In addition, rats fed a diet rich in olive oil displayed a significant suppression of NK cell activity compared to control rats fed a low-fat diet or diets containing hydrogenated coconut oil or safflower oil (Yaqoob *et al*., 1994b). However, it is important to notice that these animal studies have administrated diets enriched with large amounts of MUFA (200g/kg), contributing for approximately 30% of total energy intake (Yaqoob, 2002). Another study that used relatively small amounts of oleic acid in the diet showed an opposite effect. Thus, these animals showed increased proliferation of lymphocytes and NK cell activity (Jeffery *et al*., 1997). Thus, there have been contradictory results regarding the effects of MUFA on immune functions, which are mostly because of differences in the concentrations of fatty acid supplemented (Yaqoob, 2002).

In contrast to the animal studies, consumption of a MUFA-rich diet by healthy human individuals did not produce changes in immune cell functions (Yaqoob *et al*., 1998). Thus, middle-aged men who were randomly assigned to consume either a control diet or a diet containing foods enriched with highly refined olive oil for 8 weeks showed no change in the proliferative response of either whole blood cultures or peripheral blood mononuclear cells in response to ConA (Yaqoob *et al*., 1998). The consumption of MUFA diet also did not affect NK cell activity in humans (Yaqoob *et al*., 1998). The different results obtained in animals and humans may be attributable to the higher amount of monounsaturated fat used in the first than in the second (Yaqoob, 2002).

Roles of polyunsaturated fatty acids in the immune system

PUFA are incorporated into cell membrane phospholipids and serve as precursors of eicosanoid synthesis. Arachidonic acid (AA, C20:4 *n*-6) and eicosapentaenoic acid (EPA, 20:5 *n*-3) are converted through phospholipase A2, cyclooxygenase (COX) and lipoxygenase (LOX) to prostaglandins, thromboxanes, leukotrienes, as well as various hydroxyl-fatty acids. Resulting metabolites from these reactions are widely known as eicosanoids, which are involved in modulating the intensity and duration of inflammatory responses. Because of the relatively high amount of arachidonic acid in membrane phospholipids of cells involved in inflammation, this fatty acid is typically the major precursor for eicosanoid mediators, which are produced in high amounts upon cellular stimulation. In general, AA-derived eicosanoids are more physiologically potent and of a pro-inflammatory nature than those derived from *n*-3 PUFA, which are often much less biologically active (Calder, 2010; Lorente-Cebrián *et al*., 2013; Schmitz and Ecker, 2008). Although this idea is considered an over-simplification since it is now recognized that eicosanoids derived from arachidonic acid, such as PGE_2, has both pro- and anti-inflammatory effects (Calder, 2010). In contrast, EPA and DHA are claimed to act mainly as anti-inflammatory agents (Calder *et al*., 2010). There is a number of ways that *n*-3 PUFA seems to reduce inflammation, according to Calder (2010), these include: (1) decrease the production of eicosanoid mediators from arachidonic acid, many of which have pro-inflammatory roles, (2) increase the production of weakly inflammatory or anti-inflammatory eicosanoids from EPA, (3) increase the production of anti-inflammatory resolvins from EPA and DHA, (4) decrease the chemotactic responses of leukocytes, (5) decrease the adhesion molecule expression on leukocytes and on endothelial cells and decrease the intercellular adhesive interaction, and (5) decrease the production of pro-inflammatory cytokines and other pro-inflammatory proteins induced via NFκB system.

Given that the metabolism of the *n*-6 and *n*-3 fatty acids is competitive, an excess of fatty acid series may lead to a decrease in the conversion of the other series (Schmitz and Ecker, 2008). Thus, an excessive intake of *n*-6 PUFA (linoleic acid) decreases the formation of *n*-3 PUFA, such as

EPA and DHA, and promotes the synthesis of pro-inflammatory eicosanoids. In fact, when competing with larger amounts of *n*-6 PUFA, the conversion of α-linolenic acid into EPA is very low (from 0.2 to 6%) and the conversion into DHA is 0.05% or less (Goyens *et al*., 2006). The balance of *n*-6/*n*-3 PUFA is an important determinant in homeostasis maintenance, normal development, and mental health throughout life (Simopoulos, 2008; 2009). However, western diets contain excessive amounts of *n*-6 PUFA and very low amounts of *n*-3 PUFA, resulting in an unhealthy *n*-6/*n*-3 PUFA ratio of 20:1, instead of 1:1 found during evolution of humans (Simopoulos, 2016). This potentially could contribute to a pro-inflammatory milieu and the progression of obesity and obesity-related diseases (Lorente-Cebrián *et al*., 2013).

Several studies evaluated the effects of polyunsaturated fatty acids on immunity. However, most studies in which *n*-6 PUFA were supplemented in rodent diets showed no effect on lymphocyte proliferation (Yaqoob *et al*., 1994a; 1995a), NK cell activity (Yaqoob *et al*., 1994b) or cytokine production (Yaqoob and Calder 1995a; 1995b). In contrast, a number of dietary supplementation studies with *n*-3 PUFA (delivered as fish oil) demonstrated a time-dependent decrease in chemotaxis of human neutrophils and monocytes towards various chemoattractants (such as leukotriene B4, bacterial peptides and human serum) (Endres *et al*., 1989; Lee *et al*., 1985; Luostarinen *et al*., 1992; Schmidt *et al*., 1991; Sperling *et al*., 1993). Both the distance of cell migration and the number of cells migrating were decreased. In addition, an inhibition of both lymphoproliferation (Arrington *et al*., 2001; Jeffery *et al*., 1996; Jolly *et al*., 1997; Yaqoob *et al*., 1994a; 1995) and NK cell activity (Jeffery *et al*., 1996; Yaqoob *et al*., 1994b) were found in *n*-3 PUFA supplemented rodents. However, as previously commented in MUFA rich diets, it should be notice that these animal studies employed large amounts of fatty acids in the diet. For instance, these diets can contain as much as 200 g fish oil/kg, reaching approximately 12% of dietary energy from *n*-3 PUFA. Regarding human studies, several studies showed no effect of *n*-3 PUFA (fish oil) intake on the production of inflammatory cytokines (Cooper *et al*., 1993; Mølvig *et al*., 1991; Schmidt *et al*., 1996; Yaqoob *et al*., 2000). However, another work indicates that fish oil

supplementation inhibited both lymphocyte proliferation and NK cell activity compared to a placebo treatment in healthy subjects aged 55-75 years old (Thies *et al*., 2001). In addition, previous studies report that fish oil supplementation suppressed the production of inflammatory cytokines (such as TNF-α, IL-1 and IL-6) in humans (Caughey *et al*., 1996; Gallai *et al*., 1995).

Although some studies demonstrated immunomodulatory effects of *n*-3 PUFA, it is not yet clear whether they are associated with EPA or DHA, or a combined effect of these two *n*-3 PUFA. Animal studies suggest that both EPA and DHA have immunomodulatory effects. For instance, both EPA and DHA, fed to rats at 4.4g/100g total fatty acids, inhibited lymphocyte proliferation, although only EPA inhibited NK cell activity (Peterson *et al*., 1998). In addition, both EPA and DHA inhibited the proliferation and production of IL-2 by splenic lymphocytes in mice (Jolly *et al*., 1997). However, two animal models of inflammation demonstrated differential effects of EPA and DHA, one suggesting reduced inflammation by DHA (Tomobe *et al*., 2000), whereas the other suggests that EPA was the most anti-inflammatory (Volker *et al*., 2000). In humans, a comparison of the effects of 3.8 g EPA/day or 3.6g DHA/day, with a control treatment of linoleic acid, reported no differential effects of the *n*-3 PUFA on the phagocytic activity of monocytes (Halvorsen *et al*., 1997). Another study compared the effects of supplementation with fish oil (< 1g/day), highly-purified DHA (< 1g/day) and a placebo on lymphocyte proliferation and NK cell activity in healthy subjects and demonstrated that fish oil inhibited lymphocyte proliferation and NK activity, whereas DHA had no effect. This finding could suggest that either EPA is responsible for the inhibitory effect or that both EPA and DHA are required (Thies *et al*., 2001).

In addition, studies conducted in animals, investigating the influence of dietary fatty acids on host survival and/or pathogen clearance challenged with a live infectious agent were inconclusive, some studies showing that *n*-3 PUFA improved host defence, whereas others found suppressed immune functions (Anderson and Fritsche, 2002).

1.8. FERMENTED MILK WITH PROBIOTICS SUPPLEMENTATION AS A NUTRITIONAL INTERVENTION TO AMELIORATE THE AGING PROCESS

1.8.1. Main characteristics of the gut microbiota.

Recent data estimate that around 3.8 x 10^{13} microbial cells colonize the body of an average human male. Thus, this slightly exceeds the number of human cells in the body that constitutes the host, which are estimated to number 3.0 x 10^{13} to 3.7 x 10^{13} cells, in males (Sender *et al*., 2016). The microbial associates that reside in and on the human body constitute the microbiota, and it is mostly constituted by bacteria, but also by other microorganisms such as fungi, archaea, viruses, and protozoans (Sekirov *et al*., 2010). Their combined genomes contain millions of genes (microbiome), which are hundred times the number of human genes (Kamo *et al*., 2017). These microorganisms colonize all the surfaces of the human body that are exposed to the environment, with the majority residing in the intestinal tract (Clemente *et al*., 2012). The microbiota interacts with the host, greatly impacting human health and disease, being referred as a "forgotten organ" (O´Hara and Shanahan, 2006). The gut microbiota is involved in several host functions, including the production of micronutrients (such as essential vitamins and cofactors), transformation of xenobiotics, breakdown of complex lipids, proteins, and polysaccharides into metabolite intermediates (e.g., short-chain fatty acids), and also represents a barrier against pathogens (Picca *et al*., 2018). Importantly, the gut microbiota interacts with the immune system, providing signals to promote the maturation of immune cells and the normal development and homeostasis of the host immune system (Chow and Mazmanian, 2010). Indeed, 70% of the body immune cells reside in the gut-associated lymphoid tissue. The interaction between gastrointestinal cells and gut microbiota fosters immunological tolerance or inflammatory responses to pathogens. This crosstalk between microbiota and gut mucosal cells (enterocytes, dendritic cells, lymphocytes, macrophages, and M cells) modulates the production of various cytokines and chemokines (Picca *et al*., 2018).

In addition, the gut microbiota has an important role in the development and function of the central nervous system (CNS) and enteric nervous system (ENS) (Heiss and Olofsson, 2019).

In contrast with genomic variation between humans, which reaches minimal differences, microbiome achieves 80-90% variation between regions of the same person (García-Peña *et al*., 2017). Although the high interindividual variability, determined by height, sex, age, or place of residence, it has been determined that three specific microbial enterotypes (*Bacteroide*s, *Prevotella,* or *Rumunococcus*) are constants (Arumugam *et al*., 2011). In healthy adults approximately 80% of the total gut microbiota is integrated by the phyla *Firmicutes* and *Bacteroidetes* (García-Peña *et al*., 2017).

The gut microbiota development starts at the birth, showing instability and reduced diversity. Environmental factors include the intrauterine microbiota of the mother and birth type. In this sense, the microbiota of the infant is more or less diverse according to the birth method. Thus, the gut microbiota of babies born by caesarean section showed less diversity of bacteria, particularly less *Bifidobacteria,* than vaginal delivery (Biasucci *et al*., 2008; Grönlund *et al*., 1999). As soon as the baby starts feeding, the food source (formula or breast milk) also determines the gut microbiota. Some authors suggest that microbiota from formula fed babies is characterized by having more pro-inflammatory taxa as well as higher bacterial loads than breast milk fed babies (O´Sullivan *et al*., 2015). Gut microbiota remains unstable until the infant is 2-3 years old, and will highly depend on environmental factors such as introduction of solid food, place of residence, antibiotic exposure and genetic host factors. The microbiota matures at around 3 years old when it reaches a more stable composition, as described in adulthood (García-Peña *et al*., 2017). In particular, dietary changes have shown significant effects on the microbiota. For instance, a study showed that changing from a low-fat, plant polysaccharide-rich diet to a high-fat, high-sugar western diet is able to change the microbiota within a day (Turnbaugh *et al*., 2009). Similarly, another study in human demonstrated that the shift from a high-fat/low-fibre diet to a low-fat/high-fibre diet caused notable changes in the microbiota within a day (Wu *et al*., 2011b). Diet also seems

to correlate with enterotype, as individuals on a diet high in fat have a *Bacteroides*-dominated enterotype, whereas a carbohydrate-rich diet is associated with the *Prevotella*-dominated enterotype (Wu *et al*., 2011b).

Microbiota-gut-brain axis

Sudo *et al*. (2004) was the first to find an association between the gut microbiota and CNS, showing an increased hypothalamic-pituitary-adrenal (HPA) stress response and decreased brain-derived neurotrophic factor (BDNF) levels in the hippocampus of germ-free mice. More recently, studies found a relationship between gut microbiota and CNS-related disorders in humans, such as Parkinson´s disease and autism (Kang *et al*., 2013; Scheperjans *et al*., 2015; Tomova *et al*., 2015). A bidirectional gut-brain communication involving the microbiota comprises neural (e.g., enteric nervous system (ENS), vagus, and sympathetic and spinal nerves), humoral pathways (e.g., cytokines, hormones, and neuropeptides as signalling molecules) and the metabolites of the microbiota (Cryan and O´Mahony, 2011). This communication network is referred as the "microbiota-gut-brain axis" and signals gastrointestinal perception to the brain, which in turn elaborates a gastrointestinal response (Mayer, 2011). The microbiota is able to alter the levels of neurotransmitter precursors and to regulate the synthesis and the release of serotonin, dopamine, actylcholine, norepinephrine and γ- aminobutyric acid (GABA) (Lyte, 2013; O´Mahony, 2015). Events like psychological or physical stress can significantly dysregulate the gut-brain-axis through the action of the hypothalamic-pituitary-adrenal axis, which regulates the stress response (Scott *et al*., 2013; Wang and Kasper, 2014). An example of this pathological condition is irritable bowel syndrome (O´Mahony *et al*., 2009). In addition, through this gut-brain axis, the microbiota is able to influence numerous aspects of host health, including organ morphogenesis, immune system and gastrointestinal tract development and maturation, intestinal vascularization, tissue regeneration, carcinogenesis, metabolism, bone homeostasis, memory function, emotional arousal, affective behaviour, intuitive decision-making, and a range of neurological diseases (Picca *et al*., 2018).

1.8.2. Age-related changes in the gut microbiota

The process of aging deeply affects the composition and diversity of the gut microbiota (García-Peña *et al*., 2017; Salazar et al., 2017). In addition, the perturbation of gut microbiota (denominated dysbiosis) has been linked to chronic inflammation (Rehman, 2012) and to age-related diseases, such as neurodegenerative diseases (Friedland, 2015), cognitive decline (Magnusson *et al*., 2015), frailty (Meehan *et al*., 2015), type 1 and type 2 diabetes (Paun and Danska, 2016), non-alcoholic fatty liver disease and cardiovascular disease (Sanduzzi Zamparelli *et al*., 2016).

Age-related alterations in the gut physiology, such as gastric motility disorders, hypochlorhydria, chewing difficulties, and degenerative changes in enteric nervous system, as well as changes in diet patterns (such as increased consumption of high sugar-fat foods and reduction in plant-based foods) have great effects on the diversity and composition of intestinal microbiota (Konturek *et al*., 2015). In addition, immunosenescence and low-grade chronic inflammation may also contribute to alterations in the gut microbiota and *vice versa* (Vaiserman *et al*., 2017). Although there is a consensus that dominant species that conform microbiota in elderly people remains unchanged, it has been reported that there are significant alterations in the proportion and composition of the different taxa, leading to a decrease in the microbiota diversity (including low *Bifidobacteria* species), as well as an increase in some enteropathogens that may lead to chronic inflammation (Mueller *et al*., 2006; Rajilić-Stojanović *et al*., 2009). In addition, the *Bacteroidetes* are increased, while the *Firmicutes* appear to be lower in elderly individuals compared to younger adult controls. The elderly population also shows decreased levels of *Clostridium cluster* XIV and *Faecalibacterium Prausnitzii*, which are known as major producers of butyrate. Butyrate is a short chain fatty acid known as a source of energy for enterocytes and has been implicated in the protection against intestinal inflammatory diseases (Mueller *et al*., 2006; Rajilić-Stojanović *et al*., 2009). A previous study indicates that *Firmicutes* and *Bacteroidetes* were the dominant phyla in the gut microbiota of centenarians (Andersson *et al*., 2008; Tap *et al*., 2009). In addition, *Eubacterium*

limosum and relatives are specifically identified as signature bacteria of longevity, as they are more than 10-fold increased in centenarians. Authors suggest that at approximately 75-80 years old is the group at age at which microbiota changes with aging (Biagi *et al*., 2010; Vaiserman *et al*., 2017). In a more recent study of super centenarians (105-109 years of age), a higher prevalence of particular health-associated groups (such as *Akkermania*, *Bifidobacterium*, and *Christensenellaceae*) has been observed in these super centenarians in comparison with adults, elderly and centenarians (Biagi *et al*., 2016). Furthermore, another study demonstrated increased production of compounds with anti-inflammatory properties in the gut microbiota of centenarians, which could favour the successful aging process observed in this population (Collino *et al*., 2013).

1.8.3. Main characteristics of probiotics

The word "probiotic" is derived from the Greek meaning "for life" and it has the exact opposite meaning of "antibiotic". The immunologist Élie Metchnikoff was the first to propose the beneficial effects of probiotic microorganisms on human health. Metchnikoff hypothesized that Bulgarians were healthy and lived longer because of the consumption of fermented milk containing *Lactobacillus* spp (Gordon, 2008). According to Food and Agriculture Organization of the United Nations/ World Health Organization (FAO/OMS), probiotics are defined as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (FAO/WHO, 2002; Hill *et al*., 2014). Probiotics are classified as functional foods. These types of food are claimed to have a positive effect on health. The term "functional food" was first used in Japan, in the 1980s, for food products fortified with special constituents that have beneficial effects in the organism (Kwak and Jukes, 2001; Stanton *et al*., 2005). Such food products are gaining increasing popularity and acceptance in the world. Furthermore, increased commercial interest in exploiting the proposed health attributes of probiotics has contributed significantly to the rapid growth and expansion of this sector of the market (Saad *et al*., 2013).

The most commonly used probiotics belong to the genera *Lactobacillus* and *Bifidobacterium* (FAO/WHO, 2001; Holzapfel *et al*., 2001). However, other bacteria and some yeast (such as *Saccharomyces boulardii*) also have probiotic properties **(Table 3)**. The genus *Lactobacillus*, which belongs to the phylum *Firmucutes*, is classified as lactic acid bacteria (LAB). These include gram-positive catalase-negative bacterial species, which produce lactic acid as the main end product of the fermentation of carbohydrates. They are ubiquitously found in environments where carbohydrates are available, such as food (dairy products, fermented meat, sour dough, vegetable, fruits, beverages), respiratory, gastrointestinal and genital tracts of humans and animals. In contrast, the genus *Bifidobaterium*, which belongs to the phylum *Actinobacteria*, is only poorly phylogenetically related to genuine LAB (still they are traditionally listed among LAB) and its species use a metabolic pathway for the degradation of hexoses different from those described for "genuine" LAB. They are gram-positive, catalase-negative (with some exceptions) and occur in animal and human habitats. Also, the two species, *Streptococcus thermophiles* and *Lactococcus lactis*, which belong to lactic acid bacteria, play an important role in the production of fermented milks and dairy products. *Streptococcus thermophiles* is often used in association with *L. delbrueckii* subsp. *bulgaricus* as a starter culture for the production of yogurts. These two bacteria are associated with alleviation of symptoms of lactose intolerance and other gastrointestinal disorders (Felis and Dellaglio, 2007). It is important to mention that probiotic properties are strain specific and cannot be extrapolated to other strains, even within the same species (Pineiro and Stanton, 2007). In addition, these microorganisms in order to be considered probiotics should be normal inhabitants of a healthy intestinal tract, survive the upper digestive tract, be capable of surviving and growing in the intestine (acid and bile resistance), and safe for human intestinal cell lines and colonization (Guarner and Schaafsma, 1998; Guarner *et al*., 2005; Morelli, 2000). The mechanisms of action of probiotics against gastrointestinal pathogens are not entirely known, but some studies suggest that they are involved basically on: (1) the production of inhibitory substances, such as some organic acids, hydrogen peroxide and bacteriocins, which are inhibitory to

both gram-positive and gram-negative bacteria, (2) blocking of adhesion sites (given that probiotics and pathogenic bacteria are in a competition, probiotics seem to inhibit the pathogens by adhering to the intestinal epithelial surfaces, (3) competition for nutrients (probiotics seem to inhibit the pathogens by consuming the nutrients that pathogens need), (4) improvement of the barrier function of the epithelial lining, (5) stimulating of immunity, and (6) influence on other organs of the body through the immune system and neurotransmitter production (GABA or serotonin) (Sánchez *et al*., 2017). Many studies report effects of probiotic consumption on several health-related outcomes, including incidence and duration of gastrointestinal infections, improving vitamin synthesis, normalizing intestinal transit, alleviating lactose intolerance, diminishing incidence of allergy, prevention of urogenital diseases, and protection against colon and bladder cancers (Sharma and Devi, 2014).

Table 3. Microorganisms considered probiotics.

Lactobacillus species	***Bifidobacterium species***	***Other lactic acid bacteria***	***Non-lactic bacteria***
			Escherichia coli strain Nissle
L. acidophilus	*B. breve*	*Enterococcus faecium*	*Sacchromyces cerevisae*
L. casei	*B. infantis*	*Lactococcus lactis*	*Saccharomyces bourlardii*
L. delbrueckii subsp. bulgaricus	*B. lactis*	*Leuconstoc mesenteroides*	
	B. longum	*Pediococcus acidilactici*	
L. farciminis	*B. thermophilum*	*Streptococcus thermophilus*	
L. fermetum		*Streptococcus diadetylactis*	
L. gasseri		*Streptococcus intermedius*	
L. johnsonii			
L. paracasei			
L. plantarum			
L. reuteri			
L. rhamnosus			

Cited in Holzapfel *et al*., 2001

1.8.4. Roles of probiotics in the immune system

Increasing evidence suggests that probiotics are capable of stimulating both innate and adaptive components of the immune system, thereby conferring health benefits to the host. The

gastrointestinal tract is the main entry site of bacteria and other pathogens through dietary intake and has a potent antigen-presenting region constituted by the gut-associated lymphoid tissue with an abundance of immune cells. Probiotics interact with these cells of lymphoid tissue, which finally culminates in downstream activation of various immunological pathways. Several studies report enhanced phagocytosis and macrophage production, increased secretion of lysosomal enzymes, increased ROS production, and modified cytokine release in peritoneal and pulmonary macrophages of both animal models and human trials that received oral administration of probiotics (Habil *et al*., 2011; Ivec *et al*., 2007; Kapila *et al*., 2012; Kekkonen *et al*., 2008; Perdigón *et al*., 1998). Studies in rats and mice suggest that oral administration of lactic acid bacteria increased the numbers of T lymphocytes, CD4+ T cells, and antibody-secreting cells in the intestinal mucosa and enhanced lymphocyte proliferation (Aattour *et al*., 2001; Jain *et al*., 2009; Schiffrin *et al*., 1995). However, the exact mechanisms underlying these effects of probiotics are incompletely understood. Increasing evidence suggests that the immunomodulatory effects of probiotics are based on their ability to interact through M cells in the Peyer´s patches of intestinal epithelial lining. These mucosal epithelial cells are critical in coordinating the immune defence by releasing cytokines in response to external signals and by recruiting cells from both the innate and adaptive immune responses. Once inside the lamina propria, probiotics interact with dendritic cells and macrophages, stimulating them to produce cytokines, which results in immunomodulatory effects of probiotics. These effects include increased proliferation of CD4+ T cells, enhanced IgA production, and cytokine synthesis favouring anti-inflammatory responses (Sharma and Devi, 2014).

Roles of probiotics in immunosenescence

Probiotics are among the most actively interventions aimed at improving health status in aging (Rondanelli *et al*., 2015; Saraswati and Sitaraman, 2015). The age-related changes of the immune system or immunosenesence result in increased incidence, duration and persistence of infections in elderly individuals. In particular, the consumption of probiotics has been reported to boost immune response against various infections during aging. Thus, a previous study conducted

in the elderly population showed that the consumption of fermented milk containing cultures of yogurt and the probiotic *Lactobacillus casei* DN-114001 for 3 weeks reduced the duration of winter infections (Turchet *et al*., 2003). Also, the consumption of the same fermented milk (*Lactobacillus casei* DN-114001) in an elderly free-living population resulted in reduced duration of common infectious diseases of the airways and gastrointestinal tract (Guillemard *et al*., 2010). Moreover, the dietary consumption of yogurt fermented with *Lactobacillus delbrueckii* ssp. *bulgaricus* OLL1073R-1 resulted in significantly lower incidence of common cold (2.6 times lower). This was corroborated with higher NK cell activity in the probiotic-fed group in comparison with controls (Makino *et al*., 2010). In addition, the enterally fed fermented milk containing *Lactobacillus johnsonii* La1 (NCC533) for 12 weeks decreased the number of days with infections as well as enhanced blood phagocytic activity and suppressed the fecal antibiotic-resistant strain methicillin-resistant *Staphylococcus aureus* in hospitalized, bed-ridden elderly individuals (Fukushima *et al*., 2007). Another study indicates increased cytotoxicity and phagocytosis of peripheral blood mononuclear cells in elderly individuals after dietary supplementation with probiotic cheese containing *Lactobacillus rhamonosus* HN001 and *Lactobacillus acidophilus* NCFM. These results further concluded that daily consumption of the probiotic cheese enhanced parameters of innate immunity in elderly volunteers (Ibrahim *et al*., 2010). Also, the administration of milk supplemented with *Bifidobacterium lactis* HN019 and *Lactobacillus rhamnosus* HN001 in elderly subjects resulted in increased proportions of T lymphocytes and NK cells, as well as improved phagocytic activity (Gill *et al*., 2001). Studies in elderly individuals indicate that dietary consumption of probiotic lactobacilli stimulated the production of specific IgA and IgG antibodies in response to influenza vaccination (Boge *et al*., 2009; Bosch *et al*., 2012). Thus, these data indicate that the consumption of probiotics could help to generate an adequate immune response to vaccination in the elderly.

1.8.5. Roles of probiotics in the central nervous system

Increasing evidence suggests a role for the gut microbiota in the gut-brain axis, which has shown to influence behaviour through the central nervous system (CNS). Thus, the consumption of probiotics seems to have an important role in the gut-brain axis communication, beneficiating gut and brain functions. For instance, the consumption of probiotics in animal models, which includes *B. lungum, B. breve, B. infantis, L. helveticus, L. rhamnosus, L. plantarum, or L. casei* strains, were able to control anxiety and depression, and to improve memory related behaviours (Groeger *et al*., 2013; Lomasney *et al*., 2014; Saulnier *et al*., 2013). Doses between 10^9 and 10^{10} CFU and durations of 2 weeks in animals and 4 weeks in humans have shown beneficial effects, such as anxiety, depression, autism spectrum disorder, obsessive-complusive disorder, and memory abilities (Wang *et al*., 2016). Although the mechanisms of probiotics effects on CNS functions are largely unknown, it seems that probiotics can alter directly CNS biochemistry, through changes in the levels of BNDF, γ-aminobutyric acid (GABA), serotonin 5 hydroxytryptamine, and dopamine (Bercik *et al*., 2011; Bravo *et al*., 2011; Liu *et al*., 2016). In addition, both the vagus and the enteric nerves are involved in the gut-brain axis and can be affected by certain probiotics (Bercik *et al*., 2011; Bravo *et al*., 2011). The HPA stress response, which regulates mood and emotion, has shown to be attenuated by probiotics ingestion, decreasing corticosteroid levels (Ait-Belgnaoui *et al*., 2012). Furthermore, the immune system can be affected by probiotics, such as limiting pro-inflammatory status, which, in turn, can influence the endocrine and nervous system (Desbonnet *et al*., 2008; 2010).

1.8.6. Roles of probiotics in longevity

The consumption of probiotics has been described to have positive effects on the longevity of the host. A previous study showed increased longevity in mice supplemented with *Bifidobacterium lactis* for 11 months, possibly due to the suppression of chronic low-grade inflammation in the gut (Matsumoto *et al*., 2011). *Lactobacillus rhamnosus* CNCM I-3690 also

increased the life span in the nematode *Caenorhabditis elegans* by stimulating the innate immune response and reducing oxidative stress (Grompone *et al*., 2012). In another study, *Lactobacillus salivarius* FDB89 isolated from feces of centenarians in China extended the life span of *C. elegans* by up to 11.9% in a dietary-restriction dependent manner (Zhao *et al*., 2013). Similarly, the supplementation with *Lactobacillus gasseri* SBT2055 was effective to extend the life span *in C. elegans* by reducing oxidative stress and by stimulating the innate immune response through p38MAPK signalling pathway (Nakagawa *et al*., 2016). Also, a probiotic and synbiotic formulation impacted *Drosophila melanogaster* longevity through reduction of markers of physiological stress, oxidative stress and inflammation (Westfall *et al*., 2018).

2. JUSTIFICATION AND OBJECTIVES

The aging of the population is a global fact and consequently the number of people who reach an advanced age is significantly increasing. This fact justifies the importance of studying the inevitable process of aging, conditions that could accelerate it, as well as strategies that could ameliorate it, thus preventing age-related diseases and allowing a healthy aging. The maintenance of an adequate state of health is achieved by the proper functioning of all physiological systems of the organism, especially those of the regulatory systems, namely the nervous, endocrine and immune systems, as well as the communication between them. In contrast, the process of aging is characterized by a progressive deterioration of the regulatory system functions as well as of their communication. According to the oxidation-inflammation theory of aging, the age-related changes of the regulatory systems are mainly based on oxidative and inflammatory stresses. This theory also proposes that the immune system, due to its property of producing oxidative and inflammatory compounds, which in moderate levels are essential to carry out an effective immune response, if not well-regulated, could be involved in the rate of aging of each individual through the generation of oxidative and inflammatory stresses. In the context of the age-related changes of the immune functions, which are termed immunosenescence, our research group has shown that several of these immune cell functions as well as redox parameters can predict health status, rate of aging, and consequently the life span of mice. Moreover, the age-related changes in these parameters are similar to those observed in mice (peritoneal leukocytes) and in humans (blood leukocytes).

Our research group has been studying possible murine models of premature and accelerated aging. Along with these models, obesity has been proposed as a possible model of premature immunosenescence. Given that both aging and obesity share common features, such as impaired immune function and oxidative and inflammatory stresses, obesity could accelerate the process of aging and increases the risk of morbidity and mortality at premature ages. In fact, previous data from our research group demonstrated premature deterioration of immune cell functions in genetically and diet-induced (cafeteria) obese rats, obesity being proposed as a possible model of premature immunosenescence. Therefore, we **hypothesized** that the early and late adulthood onset

of diet-induced obesity would result in impairments of the nervous (behavioural responses) and immune system (functions and redox/inflammatory state of leukocytes) associated with oxidative and inflammatory stresses, which could finally result in premature and accelerated aging of mice.

The first overall objective of the present thesis is as follows:

1. To study the effects of diet-induced obesity onset on behaviour, immune function and redox/inflammatory state at different ages, as well as on the life span of mice.

This objective is subdivided into the following specific objectives:

1.a. To evaluate the effects of the early adulthood diet-induced obesity onset on immune function and redox/inflammatory state of adult female mice, as well as on their life span.

1.b. To evaluate the effects of the late adulthood diet-induced obesity onset on behaviour, immune function and redox state of middle-aged male and female mice, as well as on their life span.

Leptin is a pleiotropic hormone synthesized mainly by adipose tissue, which plays not only an important role in the central regulation of energy homeostasis and metabolism, but is also involved in many other important physiological roles. These include brain and bone development, neuroendocrine control, reproductive function, and immune system function. There is a temporary increase of leptin levels in rodents during the early postnatal period, which constitutes a "leptin surge". Thus, it has been described that neonatal mice and rats experience elevated levels of leptin from postnatal day (PND) 4 to PND 14, with a peak at PND 10. The physiological significance of

this “leptin surge” has been mainly studied in relation to its neurotropic role during brain development, particularly in the hypothalamus. However, nothing is known about the specific role of this neonatal leptin surge in the development and establishment of the immune system functions as well as in the redox/inflammatory state of male and female rats. Therefore, we **hypothesized** that the blockage of the neonatal leptin surge would result in impairments of the immune (functions and redox/inflammatory state), nervous and endocrine systems (redox/inflammatory state) of male and female rats at adolescent and adult ages.

The second overall objective of the present thesis is as follows:

2. To study the effects of the blockage of the neonatal leptin surge (PND5-9) on the immune function and redox/inflammatory state of male and female rats at different ages.

This objective is subdivided into the following specific objectives:

2.a. To evaluate the effects of the blockage of the neonatal leptin surge (PND5-9) on the redox/inflammatory state in the spleen, hypothalamus and white adipose tissue of peribubertal/adolescent male and female rats.

2.b. To evaluate the effects of the blockage of the neonatal leptin surge (PND5-9) on the immune function and inflammatory state in spleen leukocytes of peribubertal/adolescent male and female rats.

2.c. To evaluate the effects of the blockage of the neonatal leptin surge (PND5-9) on the immune function and redox state in spleen leukocytes of adult male and female rats.

During the process of aging, the maintenance of health depends mostly on lifestyle factors (approximately 75%), while genetics seem to affect this less (25%). Among these lifestyle factors, nutrition, such as an adequate antioxidant intake, has been proposed as very relevant to improve the functions and redox state of immune cells, especially in the elderly population. In addition, monounsaturated fatty acids (oleic acid derivatives), polyunsaturated fatty acids derived from n-3 series (eicosapentaenoic acid and docosahexaenoic acid) and probiotics have been proven effective in improving immune system functions and in exhibiting antioxidant and anti-inflammatory properties. However, little research has been developed to study the repercussion of these nutrients on the nervous (behavioural responses) and immune systems (functions and redox state of peritoneal leukocytes) particularly in the context of obesity, as a possible state of premature aging, and in chronological aging. Therefore, we **hypothesized** that these nutritional interventions (with unsaturated lipids or with fermented milk containing probiotics) would ameliorate the deteriotation of the regulatory systems, caused by obesity or by the physiological process of aging.

The third overall objective of the present thesis is as follows:

3. To study the effects of the dietary supplementations with 2-OHOA or with the combination of n-3 fatty acids (EPA and DHA) on the immune function and redox state of adult female diet-induced obese mice, as well as on their life span.

This objective is subdivided into the following specific objectives:

3.a. To evaluate the effects of the dietary supplementations with 2-OHOA or with the combination of n-3 fatty acids (EPA and DHA) on the function and redox state of leukocytes of adult female diet-induced obese mice, as well as on their life span.

3.b. To evaluate the effects of the dietary supplementations with 2-OHOA or with the combination of n-3 fatty acids (EPA and DHA) on the redox state of several organs from adult female diet-induced obese mice.

The fourth overall objective of the present thesis is as follows:

4. To study the effects of the dietary supplementation with fermented milk containing probiotics for different periods of time on behaviour, immune function and redox state of old mice, as well as on their life span.

This objective is subdivided into the following specific objectives:

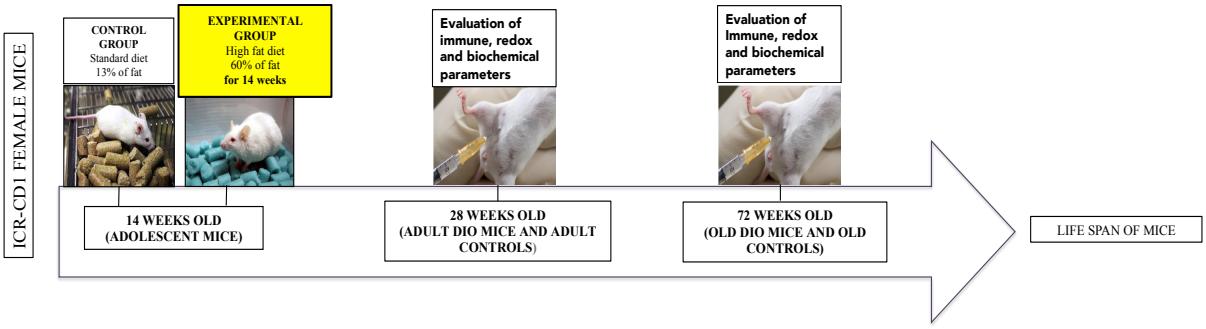
4.a. To evaluate the effects of the dietary supplementation with fermented milk containing probiotics for one and four weeks on behaviour and immune function of old mice, as well as on their life span.

4.b. To evaluate the effects of the dietary supplementation with fermented milk containing probiotics for two weeks on behaviour, immune function and redox state of old mice, as well as on their life span.

3. EXPERIMENTAL DESIGN AND RESULTS

3.1. EFFECTS OF DIET-INDUCED OBESITY ONSET ON BEHAVIOUR, IMMUNE FUNCTION AND REDOX/INFLAMMATORY STATE AT DIFFERENT AGES, AS WELL AS ON THE LIFE SPAN OF MICE

3.1.1. Effects of the early adulthood diet-induced obesity onset on immune function and redox/inflammatory state of adult female mice, as well as on their life span



Experimental design

A group of 20 adolescent mice (14±2 weeks of age) were divided into two groups of similar average body weight and were fed either a standard diet (n=10) or a high-fat diet (n=10) for 14 weeks. A group of middle-aged mice (58±2 weeks old) were fed a standard diet (n=8). The animals had free access to water and the diet. When the adolescent mice reached adulthood (i.e., the adult control and the adult diet-induced obese (DIO) mice groups, 28±2 weeks of age) and the middle-aged were considered old (i.e., the old control mice group, 72±2 weeks of age), several parameters of function and redox state of leukocytes were evaluated. In addition, adult DIO and adult control mice were submitted to the evaluation of biochemical parameters and blood pressure measurements. After 14 weeks on the high-fat diet, the adult DIO mice returned to a standard diet, while the adult control mice maintained the standard diet. When these animals reached old age (72 weeks old) (i.e., the old control and the old DIO mice groups, n=9, in each group), biochemical parameters, blood pressure measurements and peritoneal leukocyte functions were measured again.

The diets used were provided by Harlan Interfauna Iberica and the nutritional compositions were: 1) Standard diet (Teklad Global 14% Protein Rodent Maintenance Diet): Energy 2.9 Kcal/g, protein 20%, carbohydrate 67% and fat 13%, this being derived from soybean oil (saturated 0.8%, monounsaturated 0.7% and polyunsaturated fatty acids 2.1%); and 2) High-fat diet (TD.06414): Energy 5.1 Kcal/g, protein 18.4%, carbohydrate 21.3% and fat 60.3%, this fat was derived from 31% of lard and 3% of soybean oil (saturated 37%, monounsaturated 47% and polyunsaturated fatty acids 16%).

Main results

Body weight, biochemical parameters and arterial blood measurements

At adulthood (28 weeks), animals fed a high fat diet for 14 weeks displayed a significantly higher body weight when compared to animals fed a standard diet. Furthermore, they also presented higher levels of triglycerides and systolic pressure, marking them as obese. No significant differences were observed in total glucose, cholesterol and diastolic pressure levels between the diet-induced obese (DIO) mice and the control group. When these adult DIO animals reached the age of 72 weeks, they continued to present a higher body weight than the old control group. The glucose, triglycerides and systolic pressure levels were also significantly increased compared to old controls. However, with respect to measurements of total cholesterol and diastolic pressure levels, no differences were found between old control and old DIO mice groups.

Peritoneal leukocyte function parameters

The migration of peritoneal macrophages, measured by the chemotaxis index, declined significantly in adult DIO and in old control mice compared to adult controls. In addition, the number of latex beads ingested by macrophages and the number of macrophages with phagocytic ability were also significantly lower in adult DIO mice and in old controls with respect to adult control animals. The levels of intracellular superoxide anion in stimulated macrophages, an

important capacity of phagocytic cells in killing pathogens, showed significantly lower values in adult DIO mice and in old mice when compared to adult controls.

Regarding the chemotaxis capacity of peritoneal lymphocytes and the anti-tumour NK activity (% lysis), these were significantly lower in adult DIO and in old control mice with respect to adult control animals. A lower anti-tumour NK activity was also observed in both adult DIO and old control mice in comparison with adult controls. In addition, adult DIO mice and old control mice displayed a significantly suppressed mitogen-induced proliferative response compared to adult controls. However, the basal lymphoproliferation was not significantly different between the three groups of animals, adult DIO (1770±1361 cpm), old mice (1248±100 cpm) and adult controls (1459±697 cpm). The levels of a variety of cytokines (IL-1β, TNF-α, IL-6, IL-2 and IL-10) released in culture supernatants of peritoneal leukocytes after 48 h of incubation under ConA and LPS- displayed a trend towards diminution or were significantly diminished in adult DIO and in old animals in comparison with adult controls. In addition, the cytokines IL-1β and IL-10 under LPS-stimulated conditions and IL-2 under ConA and LPS- stimulated conditions were significantly lower in adult DIO mice compared to old mice. Nevertheless, the levels of IL-6 released in response to ConA and LPS were significantly higher in adult DIO mice than in old controls.

With aging, impairments of leukocyte functions became more pronounced in old DIO mice in comparison with old controls. Thus, macrophage functions, such as chemotaxis capacity as well as phagocytosis index and phagocytosis efficacy, were significantly lower in old DIO mice when compared to old controls. However, with respect to stimulated generation of superoxide anion, there were no significant differences between both groups. In relation to the anti-tumour NK activity, this was significantly lower in old DIO mice than in old controls. The lymphocyte functions (chemotaxis index and proliferation in response to ConA and LPS) were highly impaired in old DIO mice when compared to old controls. Even though not statistically significant there was a tendency of enhanced basal lymphoproliferative response in old DIO with respect to old controls.

Peritoneal leukocyte oxidative stress parameters

In order to investigate the oxidative stress status of peritoneal leukocytes in mice, the activity of xanthine oxidase, which is associated with the production of free radicals, and the enzymatic and non-enzymatic antioxidants, such as catalase activity and glutathione concentrations were evaluated. The levels of total glutathione, which have an important defensive role in neutralizing free radicals, presented lower values in peritoneal leukocytes in adult DIO and old controls as compared to adult controls. Similarly, catalase activity, which protects against oxidant compounds, was diminished in leukocytes from adult DIO and old mice with respect to adult control animals. The activity of xanthine oxidase was higher in leukocytes from adult DIO mice and in those from old mice when compared to adult controls. When these animals grew older, reaching 72 weeks of age, DIO mice exhibited significantly lower contents of total glutathione when compared to old controls. Nevertheless, the activities of xanthine oxidase and catalase did not present statistical differences between old DIO and old control animals.

Life span

Although differences were not statistically significant, DIO mice tended to have a shorter life span when compared to controls.

Partial conclusions

The results of this experiment indicate that a high fat intake during adolescence produces an obesity state, which is associated with an impaired function and redox/inflammatory state of peritoneal leukocytes in adult female mice. Thus, these results demonstrate the state of premature immnosenescence in adult DIO female mice. In addition, the early adulthood diet-induced obesity aggravates immunosenescence in old female mice.

First article regarding sub-objective 1.a.

THE GERONTOLOGICAL SOCIETY OF AMERICA®

Journals of Gerontology: Biological Sciences
cite as: *J Gerontol A Biol Sci Med Sci*, 2016, Vol. 71, No. 8, 983–991
doi:10.1093/gerona/glv082
Advance Access publication July 28, 2015

OXFORD

Original Article

Impaired Immune Response in Old Mice Suffering from Obesity and Premature Immunosenescence in Adulthood

Caroline Hunsche,* Oskarina Hernandez,* and Monica De la Fuente

Department of Animal Physiology II, Faculty of Biology, Complutense University of Madrid and Research Institute of Hospital 12 de Octubre, Madrid, Spain.

Address correspondence to Mónica De la Fuente, PhD, Department of Animal Physiology II, Faculty of Biology, Complutense University of Madrid and Research Institute of Hospital 12 de Octubre, Madrid, Spain. E-mail: mondelaf@bio.ucm.es

* These authors contributed equally to this project and should be considered co-first authors.

Received February 27, 2015; Accepted May 11, 2015

Decision Editor: Rafael de Cabo, PhD

Abstract

Obesity and aging share an impaired immune system and oxidative and inflammatory stress. Therefore, the hypothesis of obesity as a possible model of premature immunosenescence has been proposed. In this study, we investigated whether adult obese mice, as a consequence of being fed with a fat-rich diet during their adolescence, showed premature immunosenescence and if this was aggravated with aging. Peritoneal cell suspensions were obtained when ICR/CD1 obese female mice were adults (28 weeks) and old (72 weeks), and several functions and antioxidant defenses were evaluated. The results showed that the chemotaxis of both macrophages and lymphocytes, phagocytosis of macrophages, activity of natural killer cells, proliferative response of lymphocytes, interleukin-1β, tumor necrosis factor-alpha, interleukin-6, interleukin-2, and interleukin-10 released in leukocyte cultures, as well as antioxidant and oxidant capacity were significantly impaired in adult obese mice with respect to adult nonobese mice, with values similar to those in chronologically old mice. When these obese animals grew older, although having been fed with a standard diet, they showed a higher deterioration of their immune functions in comparison with the old control group. In conclusion, these results demonstrate that a high fat intake during adolescence can produce an obesity state in adult age associated with a premature immunosenescence, which is aggravated through aging.

Keywords: Obesity—Immune function—Oxidative stress—Premature immunosenescence

It is widely accepted that nutrition and diet play an important role in maintaining health, as well as in causing certain kinds of diseases. Thus, an excessive consumption of energy dense food, such as dietary fat, has been associated with the current burden of obesity worldwide (1). In particular, adolescence has been described as a critical period for the onset of obesity, and once this condition is established, it is likely to persist into adulthood (2). Furthermore, the early onset of obesity and its related health problems may increase the risk of morbidity and premature mortality (3).

Increasing evidence suggests that the central mechanism underlying obesity and its related diseases are a state of persistent low-grade systemic inflammation together with a deregulation in the inflammation–stress feedback mechanisms (4). This inflammatory condition is possibly generated by the recruitment of immune cells, mostly bone marrow–derived macrophages, into the adipose tissue in response to excess nutrients, hypertrophy, and hypoxia of adipocytes (5). Consequently, these dysfunctional adipocytes contribute to immune activation and to the increased production of several proinflammatory molecules like tumor necrosis factor-alpha (TNF-α), interleukin (IL)-6, and macrophage chemoattractant protein-1 (MCP-1) (6). Moreover, dysfunctional adipocytes seem also to be implicated in the production of oxidative stress through the generation of reactive oxygen species, which could result in further inflammation and tissue damage (7). Although an appropriate immune response requires certain levels of oxidation and inflammation, which are two interlinked processes with many feedback loops (8), several studies have shown that the enhanced inflammatory milieu observed in obesity is not associated with an optimal immune defense. Thus, obese subjects present a poorer immune response to pathogen infection and to vaccines (9,10).

In addition, it is known that the process of aging is accompanied by an impairment of the immune system, which is known as inmunosenescence. In this condition, the immune system undergoes a wide range of age-associated restructuring that may result in decreased as well as increased activities of immune cells. For instance, there is a progressive decline in the functional activity of phagocytic cells, natural killer (NK) cells, in lymphoproliferative response, and in mitogen-stimulated cytokine production (11–14). These age-related changes seem to be produced by the oxidative and inflammatory stress situation of the immune cells. In fact, although the production of low levels of oxidants and inflammatory compounds is essential for the normal defensive role of immune cells against infection, when these levels are very high, cellular damage appears. Thus, innate immune cells, such as macrophages, exhibit an age-related overactivated production of oxidative and inflammatory compounds, which occurs mainly in the absence of antigenic stimulus and cannot be neutralized by antioxidant and anti-inflammatory defenses. This leads to an oxidative and inflammatory stress (8,15–17). It is accepted that the process of aging is the result of the accumulation of cellular damage, which is caused by oxidative and inflammation stress throughout the lifetime of an organism. Moreover, it has been recently proposed that the immune system can be involved in these stresses and consequently in the rate of aging (16,17).

Because both obesity and aging share common features, such as an impaired immune system and oxidative and inflammatory stress, the hypothesis of obesity as a possible model of premature immunosenescence, has been proposed (18). Although it is known that the physiological conditions at adult age determine the aging process and that aging exacerbates obesity-induced oxidative stress and inflammation (19), the long-term immune effects of the early onset of obesity and its possible consequences in the process of aging have been scarcely investigated. Therefore, the aim of this study was to confirm whether adult obese mice, as a consequence of being fed with a high-fat diet during their adolescence, presented immune function and redox values similar to those observed in chronologically old animals, and if this premature immunosenescence is aggravated with aging.

Methods

Animals

Female ICR/CD1 mice, 8 weeks of age, were purchased from Harlan Interfauna Iberica (Barcelona, Spain). The animals were housed in polyurethane cages (6–10 animals per cage) and maintained under standard laboratory conditions (12:12 hour reversed light/dark cycle; lights on at 8:00 pm in order to avoid circadian interferences, relative humidity at 50%–60%, temperature of 22 ± 2°C, and adequate ventilation). The experiments were conducted in accordance with the guidelines and protocols of the Royal Decree 1201/2005 regarding the care and use of laboratory animals for experimental procedures and were approved by the Committee for Animal Experimentation of the Complutense University of Madrid.

Experimental Groups

A group of 20 adolescent mice (14 ± 2 weeks of age) were divided into two groups of similar average body weight and were fed either a standard diet (n = 10) or a high-fat diet (n = 10) for 14 weeks. A group of middle-aged mice (58 ± 2 weeks old) were fed a standard diet (n = 8). The animals had free access to water and food. When the adolescent mice reached adulthood (ie, the adult control and the adult obese mice groups, 28 ± 2 weeks of age) and the middle aged were considered old (ie, the old control mice group, 72 ± 2 weeks of age), several parameters of function and oxidative state of leukocytes were evaluated. In addition, adult obese and adult control mice were submitted to the evaluation of biochemical parameters and blood pressure measurements. After 14 weeks on the high-fat diet, the adult obese mice returned to a standard diet, whereas the adult control mice maintained the standard diet. All mice were marked for their individual follow-up. When these animals reached old age (72 weeks old) (ie, the old control and the old obese mice groups, n = 9, in each group), biochemical parameters, blood pressure measurements, and peritoneal leukocyte functions were measured again.

The diets used were provided by Harlan Interfauna Iberica and the nutritional compositions were (i) standard diet (Teklad Global 14% Protein Rodent Maintenance Diet): energy 2.9 Kcal/g, protein 20%, carbohydrate 67%, and fat 13%, in which fat was derived from soybean oil (saturated 0.8%, monounsaturated 0.7%, and polyunsaturated fatty acids 2.1%) and (ii) high-fat diet (TD.06414): energy 5.1 Kcal/g, protein 18.4%, carbohydrate 21.3%, and fat 60.3%, in which fat was derived from 31% of lard and 3% of soybean oil (saturated 37%, monounsaturated 47%, and polyunsaturated fatty acids 16%).

Biochemical Parameters and Arterial Blood Pressure Measurements

Glucose, cholesterol, and triglyceride levels were measured with Accutrend (Roche Diagnostics, Mannheim, Germany) using blood samples collected from the tail vein of mice. Arterial blood pressure was measured using a noninvasive pressure gauge (Panlab Non-Invasive Blood Pressure System for Rodents, Harvard). Measurements were performed three times in a stress-free environment (without light and background noise), and after subjecting the mouse to a temperature of 30°C–35°C for 20 minutes to calm the animal and avoid stress inside the holder.

Collection of Peritoneal Leukocytes

The peritoneal suspensions were obtained between 8:00 am and 10:00 am to minimize circadian variations in the immune system, without sacrificing the animals, which allowed monitoring the life spans of the mice, by a procedure previously described (20,21). Briefly, 3 mL of Hanks' solution, adjusted to pH 7.4, were injected into the peritoneum, the abdomen was massaged and the peritoneal exudate cells were collected allowing the recovery of 90%–95% of the injected volume. The peritoneal leukocytes, consisting of lymphocytes and macrophages, were counted in Neubauer chambers (Blau Brand, Germany). The suspensions were adjusted to a final concentration of 5×10^5 macrophages or lymphocytes/mL in Hanks' solution or 10^6 leukocytes/mL in Hanks' solution or complete medium (Roswell Park Memorial Institute [RPMI] 1640 enriched with l-glutamine (PAA, Pasching, Austria) and supplemented with 10% heat-inactivated (56°C, 30 minutes) fetal calf serum (GIBCO) and gentamicin (100 mg/mL, GIBCO) with or without phenol red). Macrophages and lymphocytes were identified by their morphology. The cellular viability was routinely measured using the trepan-blue (Sigma, St. Louis, MO) exclusion test, and in all cases, it was higher than 98%.

The peritoneal compartment offers the potential to study unfractionated leukocytes, which better preserve the physiological environment surrounding the immune cells in vivo. This is fundamental in studies ex vivo that try to reproduce immune cell response in vivo. In fact, this response may vary or be lost in populations of purified leukocytes (22).

Chemotaxis Assay

Chemotaxis of peritoneal leukocytes were evaluated according to a slight modification of the Boyden's method (20) consisting basically of the use of chambers with two compartments separated by a filter with a pore diameter of 3 µm (Millipore, Bedford, MA). Aliquots of 300 µL of the peritoneal suspensions, with macrophages or lymphocytes adjusted to 5×10^5 cells/mL in Hank's solution, were deposited into the upper compartment, and aliquots of 400 µL of the chemoattractant, formyl-Met-Leu-Phe (10^{-8} M) (Sigma) were put into the lower compartment. The chambers were incubated for 3 hours, and then the filters were fixed and stained. The chemotaxis index was determined by counting, using an optical microscope (100×), the total number of macrophages and lymphocytes on one third of the lower face of the filters.

Phagocytosis Assay

Phagocytosis assay of inert particles was carried out following the method described by De la Fuente and colleagues (20). Aliquots of 200 µL of the peritoneal suspensions, adjusted to 5×10^5 macrophages/mL in Hanks' medium, were incubated in migration inhibitory factor plates (Kartell, Noviglio, Italy) for 30 minutes. The adhered monolayer was washed with prewarmed phosphate buffer saline and then 200 µL of Hank's medium and 20 µL latex beads (1.09 µm diluted to 1% phosphate buffer saline) (Sigma) were added. After 30 minutes of incubation, the plates were washed, fixed, and stained. The number of particles ingested by 100 macrophages was counted using an optical microscope (100×) and expressed as phagocytic index. The percentage of macrophages, which phagocyted at least one latex bead, was also determined and expressed as phagocytic efficiency.

Superoxide Anion Production Assay

Superoxide anion production was evaluated assessing its capacity to reduce nitroblue tetrazolium (NBT, Sigma), in an equimolecular reaction, following the method described by De la Fuente and colleagues (20). Briefly, aliquots of 250 µL of peritoneal cell suspensions (1×10^6 leukocytes/mL Hank's medium) were mixed with 250 µL of NBT solution (1 mg/mL in Hank's solution) (Sigma) and with 50 µL of latex beads (1%) in stimulated samples. After 60 minutes of incubation at 37°C, the reaction was stopped, the samples were centrifuged, and the supernatants were discarded. The reduced NBT was extracted with dioxin (Merck, Darmstadt, FRG), and the absorbance of the supernatants was determined at 525 nm using a spectrophotometer. The data obtained were expressed as nmoles of NBT reduced per 10^6 leukocytes by extrapolating in a standard curve of NBT reduced with 1,4-dithoerythritol (Sigma).

Natural Killer Assay

An enzymatic colorimetric assay was carried out for cytolysis measurements of target cells (Cytotox 96 TM Promega, Boehringer, Ingelheim) based on the determination of lactate dehydrogenase enzyme using tetrazolium salts, as previously used by us in these kinds of samples (21). Aliquots of 100 µL of peritoneal leukocytes, used as effector cells, were seeded in 96-well U-bottom culture plates (Numc, Roskilde, Denmark) adjusted to 10^6 leukocytes per well in RPMI 1640 medium without phenol red. Murine lymphoma YAC-1 cells, used as target cells, were added adjusted to 10^5 cells per well. Thus, the effector/target ratio was 10:1. The plates were centrifuged at 250*g* for 4 minutes to facilitate cell contacts. After 4 hours of incubation, lactate dehydrogenase enzymatic activity was measured in 50 µL/well of the supernatants by addition of the enzyme substrate and absorbance recording spectrophotometrically at 490 nm. Three kinds of control measurements were performed: a target spontaneous release, a target maximum release, and an effector spontaneous release. The results were expressed as percentage of lysis of target cells. To determine this percentage, the following equation was used: $\%\text{lysis} = ((E\text{–ES–TS}) / M\text{–ES–TS})) \times 100$, where E is the mean of absorbances in the presence of effector cells; ES is the mean of absorbances of effector cells incubated alone; TS is the mean of absorbances in target cells incubated with medium alone; and M is the mean of maximum absorbances after incubating target cells with lysis solution.

Lymphoproliferation Assay

Following the method previously described (21), aliquots (200 µL) of peritoneal lymphocytes (10^6 cells/mL complete medium) were seeded in 96-well flat-bottomed microtiter plates (Numc, Roskilde, Denmark) and 20 µL of concanalvaline A (ConA 1 µg/mL; Sigma), a T-cell mitogen (lectin), 20 µL of lipopolysaccharide (LPS, *Escherichia coli*, 055:B5 1 µg/mL; Sigma), a B-cell mitogen, or 20 µL of complete medium (spontaneous proliferation) were added per well. The plates were incubated for 48 hours at 37°C in an atmosphere of 5% CO_2. After this time, 100 µL of culture supernatants were collected for cytokine measurements and the medium was renewed, and 0.5 µCi ^{3}H-thymidine (Du Pont, Boston, MA, USA) were added to each well. After 24 hours of incubation, the cells were harvested in a semiautomatic microharvester and retained in filter paper. Thymidine uptake was measured using a beta counter (LKB, Uppsala, Sweden). The results were expressed as ^{3}H-thymidine uptake (cpm).

Cytokine Levels

The levels of the cytokines, including growth factors (IL-2), proinflammatory (TNF-α, IL-6, and IL-1β), and anti-inflammatory (IL-10) cytokines, released into the supernatants of leukocyte cultures, after 48 hours of incubation with ConA or LPS, were measured simultaneously using a Luminex xPONENT (Milliplex Mouse Cytokine/Chemokine Panel Catalog MPXMCYTO-70K, Millipore Corp, Billerica, MA). The results were expressed as pg/mL.

Total Glutathione Levels

The total intracellular glutathione levels were measured using spectrophotometry based on a previously described method with some modifications (23). Briefly, aliquots of 1 mL of peritoneal suspension (adjusted to 10^6 leukocytes per mL in Hank's medium) were centrifuged at 1,200*g* for 10 minutes at 4°C. The pellet cells were resuspended in a medium containing 5% tricloroacetic acid (TCA, Panreac, Barcelona, Spain) in 0.01 N HCl (previously degassed with helium for a minimum of 10 minutes). This was followed by three cycles of sonication for 10 seconds (with 20 seconds rest between each cycle), keeping the sample cold. Then, the samples were centrifuged at 3,200*g* for 5 minutes at 4°C. Aliquots of the supernatants of leukocytes samples were measured using the following reaction mixture: 5,5′-dithiobis (2-nitrobenzoic acid) (6 mM, Sigma), β-nicotinamide adenine dinucleotide phosphate, reduced form (β-NADPH, 0.3 mM, Sigma), and glutathione reductase (10 U/mL, Sigma). The reaction was monitored for 240 seconds and measured using spectrophotometry at a wavelength of 412 nm. The results were expressed in nmoles/10^6 cells.

Catalase Assay

The activity of catalase (CAT) was determined following the method described by Beers and Sizer, with slight modifications introduced by us (24). The peritoneal suspension was previously adjusted to 10^6 leukocytes/mL and aliquots of 1 mL were used to perform the enzymatic assay. The cells were centrifuged at 1,076*g* for 10 minutes at 4°C and the pellets were resuspended in 50 mM phosphate buffer. Then, the samples were sonicated and centrifuged at 3,200*g* for 20 minutes at 4°C. The enzymatic assay was followed using spectrophotometry for 80 seconds at 240 nm through the decomposition of H_2O_2 (14 mM in phosphate buffer) (Merck, Germany) into $H_2O + O_2$. The results were expressed as international units (U) of enzymatic activity per 10^6 cells.

Xanthine Oxidase Assay

To study the activity of xanthine oxidase (XO), we employed the commercial kit "Amplex Red Xanthine/Xanthine Oxidase Assay Kit" (Molecular Probes). The hydrogen peroxide (H_2O_2) produced by XO reacts with horseradish peroxidase present in the reaction mixture and generates a fluorescent oxidation compound resorufin whose fluorescence is measured in a plate reader (Fluorestar Optima, BMG Labtech Biomedal, Spain). Briefly, 50 µL of peritoneal suspension adjusted to 10^6 leukocytes/mL in Hank's medium were incubated with 50 µL working solution of Amplex Red reagent (100 µM) containing horseradish peroxidase (0.4 U/mL) and xanthine (200 µM). After 30 minutes of incubation at 37°C, measurements of fluorescence were performed in a microplate reader using excitation at 530 nm and emission detection at 595 nm. Data analysis was performed with xanthine standard curves at different concentrations, the results being expressed in international milliunits (mU) of enzymatic activity per 10^6 cells.

Statistical Analysis

SPSS 10.0 (SPSS, Inc., Chicago, IL) was used for the statistical analysis of the results. The data were expressed as mean ± *SD*. Each value is the mean of the data from an assay performed in duplicate or triplicate. Normality of the samples was checked by the Kolmogorov–Smirnov test and homogeneity of variances by the Levene test. The data were statistically evaluated by the Student's *t*-test for independent samples. $p < .05$ was statistically significant and $.05 < p < .1$ was considered as a trend.

Results

Body Weight, Biochemical Parameters and Arterial Blood Measurements

At adulthood (28 weeks), animals fed with a high-fat diet for 14 weeks displayed a significantly higher body weight when compared with animals fed with a standard diet ($p < .01$). Furthermore, they also presented higher levels of triglycerides ($p < .05$) and systolic pressure ($p < .001$), marking them as obese. No significant differences were observed in total glucose, cholesterol, and diastolic pressure levels between the obese and the control group (see Supplementary Table 1). When these adult obese animals reached the age of 72 weeks, they continued to present a higher body weight than the old control group ($p < .001$). The glucose ($p < .01$), triglycerides ($p < .05$), and systolic pressure levels ($p < .01$) were also significantly increased compared with that of old controls. However, with respect to measurements of total cholesterol and diastolic pressure levels, no differences were found between old control and old obese mice groups in old age (see Supplementary Table 1).

Peritoneal Leukocyte Functions

The migration of peritoneal macrophages, measured by the chemotaxis index (Figure 1A), declined significantly in adult obese and in old control mice compared with that in adult controls ($p < .001$). In addition, the number of latex beads ingested by macrophages (Figure 1B) and the number of macrophages with phagocytic ability (Figure 1C) were also significantly decreased in adult obese mice and in old controls ($p < .001$) with respect to adult control animals. The levels of intracellular superoxide anion in stimulated macrophages, an important capacity of phagocytic cells in killing pathogens, showed significantly lower values in adult obese mice ($p < .001$) and in old mice ($p < .05$) when compared with that in adult controls (Figure 1D).

Regarding the lymphocyte functions studied, the chemotaxis capacity of peritoneal lymphocytes (Figure 1E) was significantly decreased in adult obese and in old control mice ($p < .01$) with respect to adult control animals. A decreased activity of NK cells (% lysis) was also observed in both adult obese ($p < .05$) and old control mice ($p < .01$) in comparison with adult controls (Figure 1F). The results of the proliferative capacity of peritoneal lymphocytes in response to T-cell mitogen ConA and to B-cell mitogen LPS are shown in Figure 1G and H, respectively. Adult obese mice and old control mice displayed a significantly suppressed mitogen-induced proliferative response compared with that of adult controls ($p < .001$). However, the basal lymphoproliferation was not significantly different between the three groups of animals, adult obese (1,770 ± 1,361 cpm), old mice (1,248 ± 100 cpm), and adult controls (1,459 ± 697 cpm). The levels of several cytokines (IL-1β, TNF-α, IL-6, IL-2, and IL-10), released in culture supernatants of peritoneal leukocytes after 48 hours of incubation under ConA and LPS-stimulated conditions, are shown in Figure 2. All these cytokines displayed a trend towards decrease or were significantly decreased in adult obese and in old animals in comparison with adult controls. In addition, the cytokines IL-1β and IL-10 under LPS-stimulated conditions ($p < .05$) and IL-2 under ConA- ($p < .01$) and LPS-stimulated conditions ($p < .001$) were significantly lower in adult obese mice as compared with that in old mice. Nevertheless, the levels of IL-6 released in response to ConA and LPS were significantly higher in adult obese mice than in old controls ($p < .05$).

With aging, impairments of leukocyte functions became more pronounced in old obese mice in comparison with old controls. As shown in Table 1, macrophage functions, such as chemotaxis capacity ($p < .01$) as well as phagocytosis index ($p < .01$) and phagocytosis efficacy ($p < .001$), were significantly diminished in old obese mice when compared with that in old controls. However, with respect to stimulated generation of superoxide anion, there were no significant differences between both groups. In relation to NK cell activity, there was a significant decrease in old obese mice in comparison with old controls ($p < .05$). The lymphocyte functions (chemotaxis index and proliferation in response to ConA and LPS), which are shown in Table 1, were highly impaired in old obese mice when compared with old controls ($p < .001$). Even though not statistically significant, there was a tendency of enhanced basal lymphoproliferative response in old obese with respect to old controls ($p = .06$) (Table 1).

Peritoneal Leukocyte Oxidative Stress Parameters

In order to investigate the oxidative stress status of peritoneal leukocytes in mice, the activity of XO, which is associated with the production of free radicals, and the enzymatic and nonenzymatic antioxidants, such as CAT activity and glutathione levels, were evaluated. The levels of total glutathione (Figure 3A), which have an important defensive role in neutralizing free radicals,

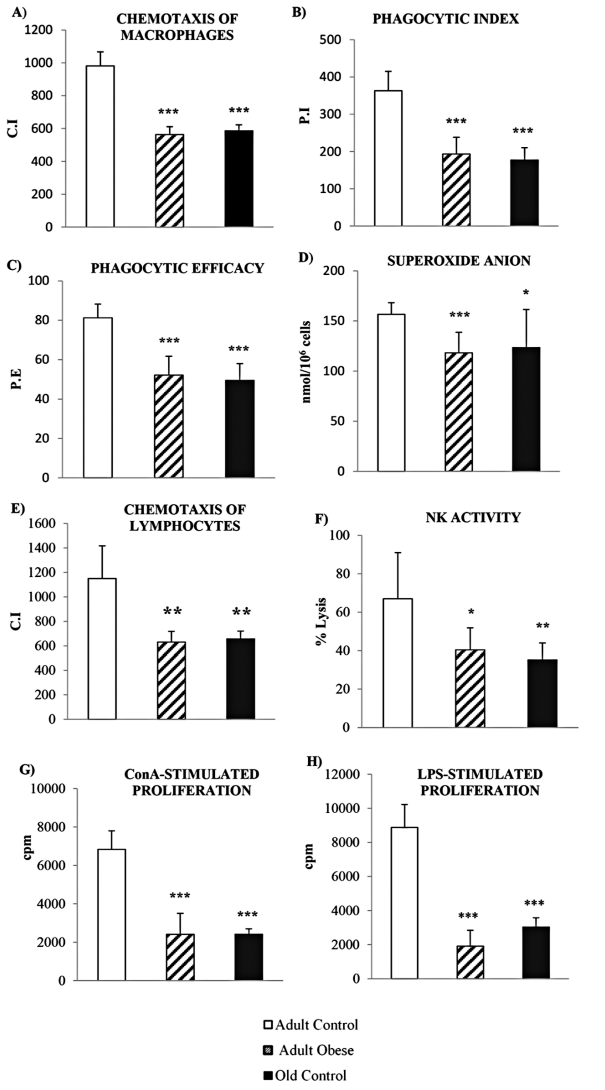


Figure 1. Immune functions. (A) Macrophage chemotaxis index (CI, number of macrophages). (B) Macrophage phagocytic index (number latex beads/100 macrophages). (C) Macrophage phagocytic efficacy (number of phagocyting macrophages/100 macrophages). (D) Stimulated intracellular superoxide anion levels (nmol/10^6 cells). (E) Lymphocyte CI (number of lymphocytes). (F) Natural killer cell activity (% lysis). (G) Lymphoproliferative response to ConA (cpm). (H) Lymphoproliferative response to lipopolysaccharide (cpm). Each column represents the mean ± *SD* of 8–10 values corresponding to that number of animals and each value being the mean of duplicate or triplicate assays. ***$p < .001$; **$p < .01$; *$p < .05$ with respect to the values of adult control mice.

presented decreased values in peritoneal leukocytes in adult obese and old controls as compared with that in adult controls ($p < .001$). Similarly, CAT activity (Figure 3B), which protects against oxidant compounds, was diminished in leukocytes from adult obese ($p < .001$) and old mice ($p < .01$) with respect to adult control animals. The activity of XO (Figure 3C) was increased in leukocytes from adult obese mice ($p < .001$) and in those from old mice ($p < .01$) when compared with adult controls. When these animals grew older, reaching 72 weeks old, obese mice exhibited significantly lower levels of total glutathione when compared with old controls ($p < .001$). Nevertheless, the activities of XO and CAT did not present statistical differences between old obese and old control animals (Table 1).

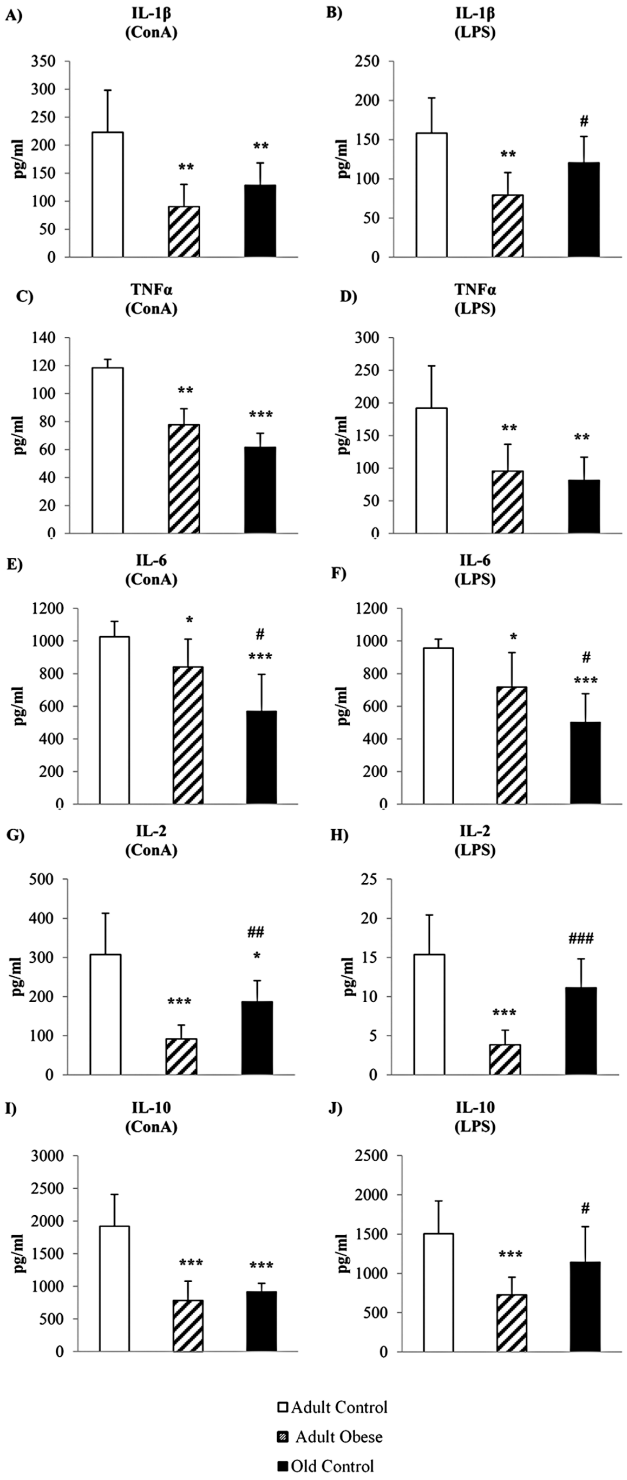


Figure 2. Cytokines levels (pg/mL) in supernatants of leukocytes cultured under ConA and lipopolysaccharide (LPS)-stimulated conditions. (A) Interleukin (IL)-1β (ConA). (B) IL-1β (LPS). (C) TNF-α (ConA). (D) tumor necrosis factor-alpha (TNF-α) (LPS). (E) IL-6 (ConA). (F) IL-6 (LPS). (G) IL-2 (ConA). (H) IL-2 (LPS). (I) IL-10 (ConA). (J) IL-10 (LPS). Each column represents the mean ± *SD* of 8–10 values corresponding to that number of animals and each value being the mean of duplicate or triplicate assays. ***$p < .001$; **$p < .01$; *$p < .05$ with respect to the values of adult control mice, ###$p < .001$; ##$p < .01$; #$p < .05$ with respect to values of adult obese mice.

Table 1. Functions and Oxidative Parameters in Peritoneal Immune Cells From Old Mice. Survival of Mice.

	Old Control Mice	Old Obese Mice
Macrophage functions		
Chemotaxis index	601 ± 50	473 ± 72**
Phagocytic index	236 ± 59	144 ± 25**
Phagocytic efficacy	64 ± 7	41 ± 9***
Stimulated intracellular superoxide anion (nmol/10^6 cells)	138 ± 57	86 ± 24
Lymphocyte functions		
Chemotaxis index	660 ± 65	507 ± 66***
Natural killer cell activity (% lysis)	50 ± 6	36 ± 11*
Basal lymphoproliferative (cpm)	1,570 ± 269	2,269 ± 873
Lymphoproliferative response to LPS (cpm)	2,795 ± 1,071	718 ± 241***
Lymphoproliferative response to ConA (cpm)	2,662 ± 1,062	887 ± 328***
Oxidative stress parameters		
Total glutathione levels (nmol/10^6 cells)	1.8 ± 0.7	0.6 ± 0.2***
Catalase activity (UI CAT/10^6 cells)	9 ± 1.2	14 ± 9
Xanthine oxidase activity (mU XO/10^6 cells)	2.7 ± 1.2	2.9 ± 0.9
Survival of mice		
Number of weeks	117 ± 11	106 ± 13

Each data represents the mean ± *SD* of 8–10 values corresponding to that number of animals and each value being the mean of duplicate or triplicate assays. *p < .05; **p < .01; ***p < .001 with respect to the values of old control mice.

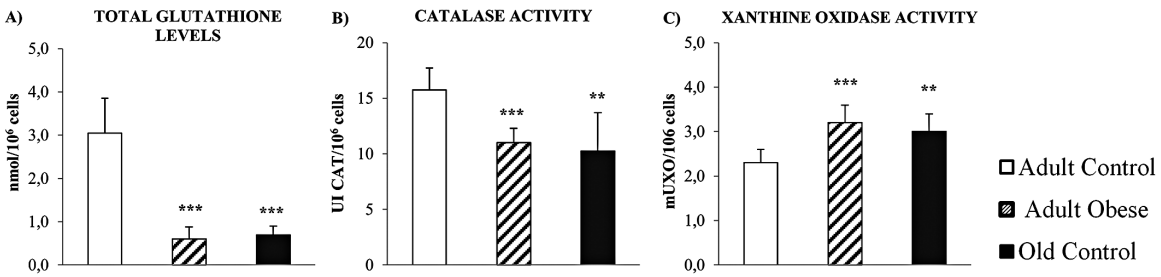


Figure 3. Oxidative stress parameters. (**A**) Total glutathione levels (nmol/10^6 cells). (**B**) Catalase activity (CAT, U CAT/10^6 cells). (**C**) Xanthine oxidase activity (mUXO/10^6 cells). Each column represents the mean ± *SD* of 8–10 values corresponding to that number of animals and each value being the mean of duplicate or triplicate assays. ***p < .001; **p < .01; *p < .05 with respect to the values of adult control mice.

Life Span

Although differences were not statistically significant, obese mice tended to have a shorter life span when compared with controls (p = 0.08) (Table 1).

Discussion

The present work shows the negative effects on the immune system of old mice as adults, were obese due to the ingestion of a high-fat diet during adolescence.

Obesity was induced in adolescent mice by the administration of a high-fat diet for 14 weeks. These animals progressively and significantly increased their body weights and also presented significantly elevated levels of triglycerides and systolic arterial pressure when compared with the control group. Moreover, these mice showed conditions associated with obesity similar to those obtained in mice of a previous study in which an identical experimental design was followed to generate adult obesity (25). Thus, the administration of a high-fat diet during adolescence seems to be a good model to induce obesity in adult mice. Additionally, this type of diet and the increased adiposity have also been reported to enhance the levels of oxidation and inflammation and to promote a dysfunctional immune response (19,26,27). Because obesity has been associated with a premature immunosenescence (18), it seems relevant to evaluate whether obesity generated at adolescence, a critical development period, could also produce some features of immunosenescence at adult age. Moreover, it is also important to know if individuals that start their aging process at adult age with premature immunosenescence reach old age with a higher deterioration of immune response than those adults showing an appropriate immune response. The present study found, on one hand, that the development of obesity during adolescence promoted a premature immunosenescence and oxidative state of leukocytes in adulthood. Thus, the adult obese mice, when compared with the controls of the same age, presented several deteriorated immune parameters, such as chemotaxis of both macrophages and lymphocytes, phagocytosis of macrophages, NK cell activity, mitogen-stimulated lymphoproliferation, and mitogen-stimulated release of several cytokines (IL-1β, TNF-α, IL-6, IL-2, and IL-10) derived from leukocytes culture supernatants. These parameters showed values similar to those in chronologically old animals. On the other hand, these adult obese mice with a premature immunosenescence reached old age in worsened conditions, although they were fed a standard diet during aging. The parameters of immune function and redox state of peritoneal leukocytes analyzed in the present study have been established as excellent markers of health and rate of aging. Moreover, it has been shown that the age-related changes in these parameters of peritoneal immune cells are similar to those in human blood leukocytes (16).

With respect to the functions studied in the peritoneal macrophages, which represent the first line of immune response, adult

obese mice showed diminished chemotaxis, phagocytosis and intracellular levels of superoxide in comparison with adult nonobese control mice. These parameters showed similar values to those in old mice. In fact, in aging individuals, an impaired migration of macrophages to the site of inflammation, a diminished phagocytosis capacity, and decreased levels of intracellular superoxide needed to kill intracellular pathogens, have been observed (12,23,28). Studies from genetic models of obesity corroborate these findings, indicating a decreased macrophage phagocytic capacity and an impaired oxidative burst in obese individuals (29,30). Morrow and colleagues (31) also reported a decreased phagocytosis by peritoneal macrophages in mice fed a high-fat diet. In addition, morbidly obese humans had a decreased bactericidal capacity by neutrophils (32). Nevertheless, another study performed in obese humans found a significantly higher phagocytosis and oxidative burst activity by monocytes and neutrophils (33). These contradictory results could be due to a different assay technique used in this study.

With respect to NK cells, also critical players of innate immunity, the data obtained in the present study showed a decreased cytotoxic capacity in old and in adult obese mice compared with that in adult controls. Previous research has indicated that aged individuals, despite their increased number of NK cells, present depressed NK cytotoxicity (12,14). Obesity also seems to produce an impaired NK cell activity. In agreement with our study, earlier findings have indicated that obese rats fed a cafeteria diet and obese mice fed a high-fat diet, as well as obese human subjects, suffer from a diminished NK cell cytotoxicity (18,27,31,34). Moreover, another study demonstrated that obese mice fed with a high-fat diet exhibited a poor cytotoxicity against influenza infection and increased mortality (35).

In addition, immune functions driven by T and B lymphocytes, such as migration and proliferation, also were affected by obesity. In fact, the present results showed that both adult obese and old mice presented similar deteriorated values in relation to peritoneal lymphocyte functions, including chemotaxis and proliferation in response to T- and B-cell mitogens such as ConA and LPS, respectively, in comparison with adult controls. There is evidence showing that aged individuals exhibit impaired chemotaxis and, especially, proliferative response to mitogens of lymphocytes (12,36). In relation to obese individuals, we did not find studies that evaluated chemotaxis of lymphocytes. Therefore, our study seems to be the first to describe a decrease in this activity in obese mice. In relation to the proliferative response of lymphocytes to mitogen stimulation, studies have indicated a reduced capacity of this parameter in obese individuals. In obese mice, Sato Mito and colleagues (37) found a decreased splenocyte proliferative response with T- and B-cell mitogens. Moreover, obese humans also exhibited suppressed stimulated proliferation of T and B lymphocytes (33,38). Increasing evidence suggests that the conditions of aging and obesity are accompanied by overactivation of innate immune cells with increased production of proinflammatory cytokines, such as TNF-α, IL-6, and IL-1β, especially in basal conditions (13,39–42). However, a previous study showed decreased levels of IL-1β, TNF-α, IL-6, IL-2, and IL-10 in peritoneal leukocytes from aged mice under mitogen-stimulated states (13). The data of the present study agree with those results. Thus, following stimulation with mitogens ConA and LPS, the secretion of these cytokines presented a decrease (tendency or statistically significant differences) in old and adult obese mice in comparison with adult controls. These findings are in agreement with those obtained in obese mice, in which peritoneal macrophages, in response to a bacterial infection, exhibited lower levels of the proinflammatory cytokines TNF-α, IL-β, and IL-6 (42–44). A decrease in spleen cells of IL-6 under LPS-stimulated conditions (45) and of the IL-2 following mitogen stimulation (46) in obese with respect to lean human individuals have also been shown. Nevertheless, in young-adult overweight human subjects, the generation of IL-2 by mononuclear or lymphocyte cells in response to LPS increased, whereas the levels of IL-10 were similar between obese and lean individuals (47). One possible explanation for these differences could be that individuals were considered mildly obese and thus might not produce significantly impaired immunity. The data of the present study also demonstrate that the release of mitogen-stimulated IL-2 was further impaired in adult obese mice with respect to old controls. Among several factors that modulate the release of cytokines, prostaglandin E2 (PGE2) has been described to inhibit the release of IL-2 (48). Although this inflammatory mediator is increased in both processes of aging and obesity (49,50), a recent study reported that adipocytes from young obese mice displayed higher levels of PGE2 with respect to old mice (51). Therefore, it is possible that the release of IL-2 was more suppressed in adult obese mice by higher levels of PGE2 than in old controls.

Oxidative stress, which is generated by an increase of oxidants and a decrease of antioxidants, has been linked to aging and obesity (16,52,53). In fact, oxidative stress seems to contribute to the pathogeneses of several diseases that are common to both aged and obese individuals, including diabetes mellitus, cardiovascular diseases, and cancer (54). The results demonstrate that old and adult obese mice exhibited elevated XO activity (an enzyme that produces oxidants) and decreased CAT activity and glutathione levels (two relevant antioxidant defenses). Previous studies have confirmed that peritoneal leukocytes from old mice or from prematurely aging mice showed enhanced activity of XO (8,55) and a decrease of enzymatic and nonenzymatic antioxidants, such as CAT activity and glutathione levels (12,16,24). In obese individuals, an increased production of oxidants and a decreased capacity of antioxidants have also been observed. Saiki and colleagues (56) reported increased levels in the blood of serum hypoxanthine and uric acid in obese compared with those in nonobese individuals, whereas Chiney and colleagues (57) found that XO activity was significantly elevated in obese when compared with that in nonobese children. Regarding antioxidant defenses, a decrease of glutathione levels in erythrocytes of obese individuals during their childhood and adulthood was reported (58–60). In addition, CAT activity was significantly lower in erythrocytes of obese women (61). Therefore, these data indicate that obesity might produce a similar oxidative stress to those reported in the elderly, and this condition could accelerate the aging process in obese individuals.

Surprisingly, when obese animals grew older, although they were fed a standard diet during their aging process, they continued to exhibit a significant higher body weight than old controls. Biochemical parameters, such as glucose and triglycerides, as well as systolic blood pressure levels, were also significantly increased in old obese mice. In addition, these animals, which maintained obesity during their aging process, showed an increased deterioration of the immune and oxidative parameters, with respect to old controls. This aggravated immunosenescence was reflected in the life span of mice, although not statistically significant, obese mice tended to exhibit a shorter life span. The consumption of a standard diet across aging was not able to restore functions and the redox state of the immune cells to the normal levels of the corresponding chronological age.

In conclusion, the results of this study provide evidence that a high-fat intake during adolescence can produce an obesity state in

adult age associated with a premature immunosenescence, which is aggravated through aging although individuals ingest a normal diet. It is possible that intervention with lifestyle strategies, such as a caloric restriction diet or the administration of appropriate amounts of antioxidants, as well as physical exercise and environmental enrichment, among others, which are effective in improving immune function in aging (62,63), could decrease the deterioration of obese mice. Thus, further studies are needed to corroborate how inadequate nutrition at an early age can influence the immune response in adulthood and then accelerate the aging process, as well as if some of the previously mentioned lifestyle strategies could prevent or delay these effects.

Supplementary material

Please visit the article online at http://gerontologist.oxfordjournals.org/ to view supplementary material.

Funding

This work was supported by grants from the Ministry of Science and Innovation (BFU2011-30336), the Research Group of Madrid Complutense University (910379ENEROINN), and Red Temática de Investigación Cooperativa en Envejecimiento y Fragilidad (RETICEF RD12/0043/0018) of Instituto de Salud Carlos III—Fondo Europeo de Desarrollo Regional (ISCIII-FEDER) from Spain.

Acknowledgments

H.C. is the recipient of a PhD fellowship from CNPq-Brazil.

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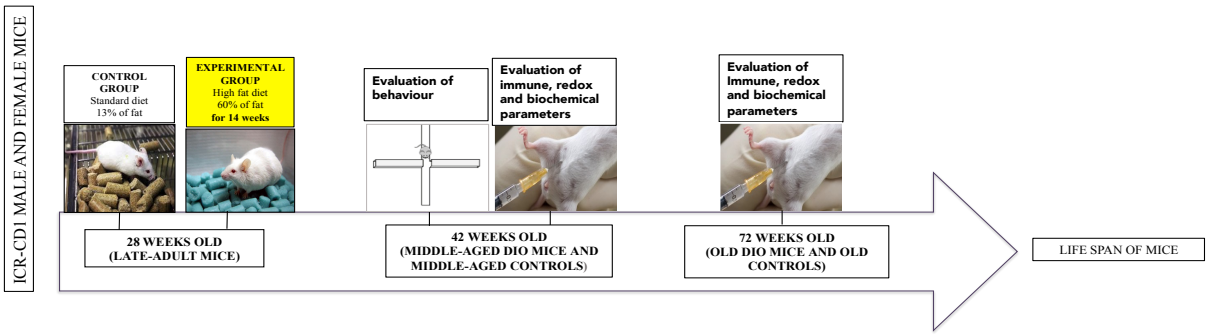
3.1.2. Effects of the late-adulthood diet-induced obesity onset on behaviour, immune function and redox state of middle-aged male and female mice, as well as on their life span.



Experimental design

Late-adult male and female mice, at the age of 28 weeks, were divided into two groups (n=8-10) each of similar average body weight and were fed either a standard diet or a high-fat diet for 14 weeks. The diets used were the same as those provided in the previous sub-objective. When male and female mice were diet-induced obese (DIO) and middle-aged (42 weeks of old), a variety of tests and assays were performed in order to evaluate behaviour, biochemical parameters as well as functions and redox state of peritoneal leukocytes. After 14 weeks on the high-fat diet, middle-aged male and female DIO mice returned to a standard diet. During the entire study, all animals had free access to water and the diet. Weight and food intake were measured every week throughout the study. Given that mice are capable of remembering previously performed behavioural tests, only biochemical parameters and the functional and redox states of peritoneal leukocytes were repeated in old age (72 weeks old). Moreover, since a significant number of male mice did not reach old age, these parameters were assessed only in female mice at the age of 72 weeks.

Main results

Body weight, total fat mass, food intake, and biochemical measurements

After the period of 14 weeks on a high-fat diet, middle-aged male and female diet-induced obese (DIO) mice displayed significantly higher body weights than their control counterparts, with males weighing more than females. In addition, male and female DIO mice consumed significantly more kilocalories than their controls, with males consuming more kilocalories than females.

Middle-aged male and female DIO mice showed a higher total fat mass than the corresponding controls, with female DIO mice showing a higher total body fat than male DIO mice. Female DIO mice showed higher values of glucose than their respective non-DIO counterparts. In addition, males displayed significantly higher levels of glucose than females. The levels of triglycerides and cholesterol were significantly higher in male and female DIO mice in comparison with their respective non-DIO controls. In addition, male DIO mice showed higher cholesterol levels than female DIO mice. When females reached 72 weeks of age (old age), they exhibited higher levels of glucose than middle-aged females.

Behavioural parameters

Motor coordination and equilibrium

The percentage of falls from the wood rod was significantly higher in male DIO mice than in their respective non-DIO controls. In addition, male DIO mice displayed significantly lower percentages of test completion and the covering of one segment in the wood rod test than male non-DIO mice. No differences were observed in these parameters between female DIO mice and their respective non-DIO controls. In the wood rod test, male DIO mice showed a worse performance in the percentage of falls, on test completion and the covering of one segment than female DIO mice.

Muscular vigour

Male and female DIO mice showed significantly higher percentages of falls from the rope than their respective female non-DIO controls. In addition, the percentages of the test completed and maximum traction capacity were significantly lower in male and female DIO mice in

comparison with their respective controls. Male DIO mice showed a worse response in the percentage of falls, in the test completed, and traction capacity than female DIO mice.

Vertical exploratory activity

The total number of rearings in the corner and holeboard tests was significantly lower in male and female DIO mice than their respective non-DIO controls. Male control and DIO mice showed a lower total number of rearings than female control and DIO mice, respectively.

Horizontal exploratory activity

Female DIO mice visited a lower number of corners in the corner test than their respective controls. Male DIO mice performed a lower number of total line crossings than their respective controls. Male DIO mice displayed a significantly lower peripheral ambulation (which was measured by the number of line crossings in the peripheral area of the holeboard) than their respective controls. Male controls also showed a significantly higher peripheral ambulation than female controls. In addition, male and female DIO mice showed a significant lower central ambulation (which were measured by the number of line crossings in the central area of the holeboard) than their respective non-DIO controls.

Male and female DIO mice took a longer time to complete the exploration of the three arms of the T-maze test than their non-DIO counterparts, with male DIO mice showing a longer time to complete the test than female DIO mice.

Anxiety-like behaviour

Male and female DIO mice exhibited a lower percentage of time in open arms than their respective non-DIO controls, with male DIO mice showing a lower percentage of time than female DIO mice. Moreover, the percentage of time in closed arms was higher in male DIO mice in comparison with their respective controls and female counterparts. In addition, the time (in seconds) spent by mice performing self-grooming in the holeboard test was significantly higher in male and female DIO mice than in their respective controls.

Male and female DIO mice also showed a higher percentage and total number of fecal bolei present than their respective non-DIO controls, with male DIO mice having a higher percentage of the presence of fecal bolei than female DIO mice.

Peritoneal leukocyte function parameters

The migration of peritoneal macrophages in response to a chemotactic gradient (formylated peptide) was significantly lower in male and female DIO mice compared with their respective non-DIO controls. Male control and DIO mice displayed a significantly lower chemotactic index (CI) than female control and DIO mice, respectively. In addition, the phagocytic index was lower in male and female DIO mice with respect to the corresponding controls, with males displaying a lower phagocytic index than females. The phagocytic efficacy was also lower in male and female DIO mice with respect to the corresponding controls.

With aging, the chemotactic index (CI) was significantly lower in old female DIO mice than in their corresponding non-DIO mice, with old females displaying a lower CI than middle-aged females. In addition, the phagocytic index (PI) was significantly lower in old female DIO mice than in non-DIO mice, with old females displaying a lower PI than middle-aged females. The phagocytic efficacy (PE) was significantly lower in old female DIO mice than in their corresponding controls, with old females displaying a lower PE than middle-aged females.

The CI of peritoneal lymphocytes was significantly lower in male and female DIO mice in comparison with their non-DIO counterparts. Male control mice displayed a lower CI than female control mice. The NK activity was significantly lower in male and female DIO mice in comparison with their non-DIO controls. Male and female DIO mice displayed significantly higher basal lymphoproliferation in comparison with non-DIO controls, with male DIO mice showing higher basal lymphoproliferation than female DIO mice. However, in response to the mitogen ConA, the proliferation of lymphocytes was lower in male and female DIO mice in comparison with their controls. Male and female DIO mice also showed lower LPS-stimulated proliferation in comparison

with their non-DIO mice, with male DIO mice showing higher LPS-stimulated proliferation than female DIO mice.

With aging, the chemotactic index (CI) of peritoneal lymphocytes in old female DIO mice was significantly lower when compared with their corresponding non-DIO mice, with old females displaying lower CI than middle-aged females. Old females showed significantly lower anti-tumour NK activity than middle-aged females. The basal proliferation of lymphocytes in old female DIO mice was higher than in their respective controls, with old females displaying higher basal lymphoproliferation than middle-aged females. Nevertheless, old female DIO mice exhibited significantly lower proliferation of lymphocytes in response to ConA than old female non-DIO mice. In addition, old females displayed significantly lower LPS-stimulated lymphoproliferation than middle-aged females.

Peritoneal leukocyte oxidative stress parameters

The catalase (CAT) activity was lower in leukocytes of male and female DIO mice in comparison with their corresponding non-DIO controls. The glutathione peroxidase (GPx) activity in leukocytes of male and female DIO mice was higher in comparison with their respective controls. Male control mice showed lower GPx activity than female control mice. The reduced glutathione (GSH) concentrations were lower in peritoneal leukocytes of male and female DIO mice in comparison with their respective non-DIO controls.

With aging, old female control mice displayed significantly lower CAT activity than middle-aged female control mice. Old females also exhibited lower GPx activity than middle-aged females. Regarding the GSH concentrations, old female DIO mice displayed lower values than those of old female non-DIO mice, with old females showing lower concentrations of GSH than middle-aged females.

The activity of xanthine oxidase (XO) was significantly higher in male and female DIO mice in comparison with their corresponding non-DIO controls, with males showing higher XO activity than females. The superoxide anion concentrations and the GSSG/GSH ratios (an indicator

of oxidative stress) were significantly higher in male and female DIO mice than in their respective non-DIO controls. The malondialdehyde (MDA) concentrations, which are an indicator of lipid oxidation and oxidative damage in cells, were significantly higher in male and female DIO mice with respect to their non-DIO controls, with male DIO mice showing higher MDA concentrations than female DIO mice.

The xanthine oxidase activity in old female controls was significantly higher than in middle-aged female controls. The MDA concentrations were significantly higher in old female DIO mice than in their respective controls, with old female controls showing higher MDA concentrations than middle-aged controls. The superoxide anion concentrations were significantly higher in old female DIO mice in comparison with old female non-DIO controls, with old female controls showing higher superoxide anion amounts than middle-aged controls.

Life span

Male DIO mice showed a shorter life span than male non-DIO control mice. Male DIO mice also exhibited a shorter life span than female DIO mice. The differences were not statistically significant between female DIO mice and female non-DIO mice.

Partial conclusions

The results of this experiment indicate that the high intake during late adulthood produces an obesity state, which is associated with impaired nervous (behavioural responses) and immune systems (function and redox state of peritoneal leukocytes) in middle-aged male and female mice. Sex differences are found in some of the behavioural, immune cell function and redox state parameters, males being significantly more affected than females. In addition, the late adulthood diet-induced obesity partially aggravates immunosenescence in old female mice.

Second article regarding sub-objective 1.b.

Brain, Behavior, and Immunity xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Brain, Behavior, and Immunity

journal homepage: www.elsevier.com/locate/ybrbi

Impacts of the late adulthood diet-induced obesity onset on behavior, immune function, redox state and life span of male and female mice

Caroline Hunsche, Irene Martínez de Toda, Mónica De la Fuente*

Department of Genetics, Physiology and Microbiology, Faculty of Biology, Complutense University of Madrid and Research Institute of Hospital 12 de Octubre (i+12), Spain

ARTICLE INFO

Keywords:
Late adulthood diet-induced obesity onset
Behavior
Immune function
Redox state
Sex differences
Life span

ABSTRACT

The aim of the present study was to investigate whether the late onset of diet-induced obesity (DIO) in middle-aged mice affected behavioral, immunological and oxidative stress parameters as well as life span of male and female mice. Also, it was analyzed whether the late DIO onset aggravated immunosenescence in old female mice. Late-adult male and female ICR/CD1 mice (28 weeks old) were fed either a high-fat diet or a standard diet during 14 weeks. After that, in these middle-aged (42 weeks old) diet-induced obese (DIO) and non-DIO controls, behavior as well as functions and redox state of peritoneal leukocytes were evaluated. These same parameters (excepting behavioral tests) were repeated when female mice were old (72 weeks old). The results showed lower exploratory activity and higher anxiety-like behavior in middle-aged male and female DIO than in controls. Moreover, these DIO animals from both sexes exhibited statistically significant impaired immune cell functions, such as chemotaxis of macrophages and lymphocytes, phagocytosis of macrophages, natural killer activity and lymphoproliferation in response to ConA and LPS, as well as an oxidative stress state in comparison with controls. Male DIO mice exhibited higher impairments in a variety of the evaluated parameters and a shorter life span than their female counterparts. In addition, female DIO mice, at old age, showed aggravated immunosenescence. In conclusion, the late DIO onset leads to impairments in behavior as well as in immune system functions of middle-aged male and female mice, males being significantly more affected than females.

1. Introduction

Unhealthy dietary patterns, such as increased consumption of diets high in fat and energy, have been associated with the worldwide obesity epidemic. The prevalence of obesity has been rising at alarming rates in all age groups (from young to older ages) and in both sexes (Johnson et al., 2015; Swinburn et al., 2011).

Obesity has been linked to systemic chronic oxidative stress, low-grade inflammation and dysfunctional immune system (Hunsche et al., 2016; Huttunen and Sryjänen, 2013; Rath and Haller, 2011; Sheridan et al., 2012). This condition seems to be generated and maintained in the adipose tissue by excess of nutrients, reactive oxygen species (ROS) and inflammatory compounds, which in turn promotes increased infiltration of immune cells and results in a vicious cycle of oxidation and inflammation. Both chronic oxidative stress and inflammation, which are two closely related processes (Vida et al., 2014), in adipose tissue, may spread to a systemic level, contributing to the development of obesity-related complications (de Mello et al., 2018). It is largely known that a controlled immune response with adequate production of inflammatory and oxidative compounds is essential for maintaining health and protecting against infections, cancer and damages. However, if the levels of these compounds exceed the capacity of anti-inflammatory and antioxidant defenses of the organism, an imbalance occurs and oxidative and inflammatory stresses are established, which contribute to cellular and tissue damage in all physiological systems, including the nervous and immune systems (Bauer and De la Fuente, 2016; De la Fuente and Miquel, 2009; Vida et al., 2014). Moreover, given that the nervous and immune systems share a bidirectional communication (Vida et al., 2014), it is expected that the dysfunctional immune system associated with obesity, would have an impact on the nervous system. In fact, increasing evidence suggests that obese individuals show increased infiltration of oxidative and inflammatory compounds into the brain, and the neuroinflammation generated has been related with the development of cognitive, motor and behavioral dysfunctions (Pistell et al., 2010; White et al., 2009).

Similar to obesity, the aging process affects functions of all

* Corresponding author at: Department of Genetics, Physiology and Microbiology, Faculty of Biology, Complutense University of Madrid, José Antonio Novais Street, 12, 28040 Madrid, Spain.
E-mail address: mondelaf@bio.ucm.es (M. De la Fuente).

https://doi.org/10.1016/j.bbi.2019.01.010
Received 15 August 2018; Received in revised form 14 January 2019; Accepted 14 January 2019

physiological systems, including those of the nervous and immune systems (Vida et al., 2014). Thus, there is a progressive deterioration in a variety of brain and behavioral functions, including memory as well as cognitive and motor activity (Rosenzweig and Barnes, 2003; Vandervoort, 2002). In addition, the immune system also suffers age-related alterations (immunosenescence), which includes marked changes of the innate and adaptive immune responses. This immunosenescence results in impaired migration and phagocytosis of macrophages, anti-tumor activity of natural killer (NK) cells and proliferative response of lymphocytes (Martínez de Toda et al., 2016). These impairments seem to be caused mainly by the age-related chronic oxidative and inflammatory stresses of the immune cells, which are also involved in the oxidative and inflammatory state of the organism and, consequently, in the rate of aging (De la Fuente and Miquel, 2009).

Given that obesity and aging are linked to oxidative and inflammatory stresses, and both conditions result in health complications, there is an increasing concern whether obesity could accelerate the process of aging and increases the risk of morbidity and mortality at premature ages (De la Fuente and Castro, 2012; Zamboni et al., 2005). We have recently shown that the early adulthood diet-induced obesity onset resulted in premature features of aging (such as immunosenescence). Thus, adult diet-induced obese (DIO) mice, as a consequence of being fed during their adolescence with a high-fat diet, presented functions and oxidative stress state of peritoneal leukocytes similar to those observed in chronologically old mice. Furthermore, when these adult DIO animals reached old age, they continuous to show higher impairments of immune cell functions, and a shorter life span than old non-DIO animals (Hunsche et al., 2016).

Previous studies indicate that the age of obesity onset (Boitard et al., 2012) and sex (Salinero et al., 2018) have an impact on obesity-related health problems. However, nothing is known about the impacts of the late adulthood diet-induced obesity onset in the nervous (behavior) and immune system (immune functions and redox state of peritoneal leukocytes) of middle-aged mice, as well as if there are differences between males and females. Therefore, in the present study, we investigated whether middle-aged male and female DIO mice, as a consequence of being fed with a high-fat diet during their late adulthood, showed alterations in relevant parameters of behavior, immune and redox state as well as in their life span. Also, it was analyzed whether the late adulthood obesity onset aggravated immune and redox state parameters of old female mice.

2. Materials and methods

2.1. Animals

Male and female ICR/CD1 mice (*Mus musculus*) were purchased from Janvier SAS (Chassal, France) at the age 8 weeks. The animals were housed in polyurethane cages (4–5 animals per cage) and maintained under standard laboratory conditions (12:12 h reversed light/dark cycle; lights on at 8:00 pm, relative humidity of 50–60%, temperature of 22 ± 2 °C and adequate ventilation). The experiments were conducted in accordance with the guidelines and protocols of the Royal Decree 53/2013 regarding the care and use of laboratory animals for experimental procedures, and were approved by the Committee for Animal Experimentation of the Complutense University of Madrid.

2.2. Experimental groups

Late-adult male and female mice, at the age of 28 weeks, were divided into groups (8–10 animals for each group) of similar average body weight and were fed either a standard diet or a high-fat diet for 14 weeks. A table with detailed nutritional content of diets is provided in a previously published paper (Hunsche et al., 2018). Briefly, the diets used were provided by Harlan Interfauna Iberica (Barcelona, Spain) and the nutritional compositions were (i) standard diet (Teklad Global 14% Protein Rodent Maintenance Diet, reference 2014): energy 2.9 Kcal/g, protein 20%, carbohydrate 67%, and fat 13% and (ii) high-fat diet (Teklad Custom Diet, reference TD. 06414): energy 5.1 Kcal/g, protein 18.4%, carbohydrate 21.3%, and fat 60.3%. When male and female mice were diet-induced obese (DIO) and middle-aged (42 weeks of old), a variety of tests and assays were performed in order to evaluate behavior, biochemical parameters as well as functions and redox state of peritoneal leukocytes. After 14 weeks on the high-fat diet, middle-aged male and female DIO mice returned to a standard diet. During the entire study, all animals had free access to water and food. Body weight and food intake were measured every week throughout the study.

Given that mice are capable of remembering previously performed behavioral tests, only biochemical parameters and functional and redox states of peritoneal leukocytes were repeated in old age (72 weeks old). Moreover, since a significant number of male mice did not reach old age (3–6 animals for each group), these parameters were assessed only in female mice at the age of 72 weeks (5–8 animals for each group).

2.3. Total body fat mass

Nuclear magnetic resonance (NMR) was used to evaluate the total fat mass in mice. NMR was performed using magnetic resonance imaging (MRI) technology Biospec BMT 47/40 with a field of 4.7 Tesla. MRI was obtained *in vivo* and analyzed (acquisition, reconstruction, and data processing) using advanced digital technology with imaging software. During the measurements, mice were anaesthetized with isoflurane (Judex et al., 2010).

2.4. Biochemical parameters

Glucose, triglyceride, and total cholesterol levels were measured with Accutrend (Roche Diagnostics, Mannheim, Germany) using blood samples collected from the tail vein of mice.

2.5. Behavioral tests

Behavioral tests were carried out between 8 am and 10 am during three consecutive days, under illumination of fluorescent lamps (20 W). On the first day, a battery of tests (reflexes, corner, T-maze, wood rod and tightrope tests) was performed in mice. On the second day, mice were subjected to the holeboard test, and on the third day mice were assessed in the elevated plus maze. The experimental apparatus was cleaned before the performance of each mouse in order to avoid olfactory trails.

2.5.1. Reflexes

When the mouse was suspended by the tail and lowered toward a solid black surface, the visual placing reflex was evaluated by observing the complete extension of the forelimbs, and the hindlimb reflex was measured by the complete extension of these limbs. The mean response was rated in three trials.

2.5.2. Motor coordination and equilibrium abilities

In order to assess motor coordination and equilibrium, animals were placed in the center of a wood elevated rod for one trial of 60 s (Baeza et al., 2010). Motor coordination and equilibrium were measured by the time (in seconds) to leave the first segment and by the percentage of mice falling off the rod. In addition, the percentages of mice that cover at least 1 segment and that complete the test were also considered as motor coordination and equilibrium abilities.

2.5.3. Muscular vigor and traction

Muscular vigor and traction were evaluated in an elevated horizontal tightrope, using a test trial of 60 s (Baeza et al., 2010). Muscular vigor was assessed as the percentage of mice falling off the rope and that complete the test. Traction was evaluated by analyzing the

different parts of the body that mice used to remain hanged (forelimbs, hindlimbs and tail) and, subsequently, the percentage of mice displaying the maximum traction capacity (using forelimbs, hindlimbs and tail).

2.5.4. Vertical exploratory activity

The vertical exploratory activity (studied as the performance of "rearing", that is, when the mouse stands up on his hindlimbs so that his body becomes more perpendicular to the ground) was evaluated in the corner test and in the holeboard test. The corner test consists in placing the mouse in a new cage with bedding during 30 s (Baeza et al., 2010). In contrast, the holeboard test consists in placing the animal in a box made of wood with matte-painted metallic walls, divided into 36 squares, bearing four equally spaced holes in the central area (all but 20 peripheral squares were considered central) (Baeza et al., 2010). The test was performed during 5 min.

2.5.5. Horizontal exploratory activity

Horizontal exploratory activity was assessed as the number of corners of the cage visited (corner test) and as the peripheral ambulation (number of line crossings in the peripheral area, by the walls) and central ambulation (number of line crossings in the central area) in the holeboard test. Peripheral ambulation was considered lower exploratory activity than central ambulation due to the tendency of mice to remain close to walls (thigmotaxis) (Simon et al., 1994). In addition, horizontal exploratory behavior was also recorded using the T-maze test, which is a wooden apparatus with three enclosed arms (Baeza et al., 2010). The mouse was placed inside the "short" arm of the maze with its head facing the wall. The time (in seconds) to complete the test (exploration of all three maze arms) was assessed as horizontal exploratory activity.

2.5.6. Goal-directed exploratory behavior

The goal-directed exploratory behavior of mice was assessed in the holeboard test. A small plastic object was placed into each of the four holes of the holeboard to attract mice attention. The frequency (percentage and number) of mice that perform head-dips (when the animal places its head into the hole) was considered as a goal-directed exploratory behavior.

2.5.7. Anxiety-like behavior

The anxiety-like behavior was measured in the elevated plus maze, as previously described (Garrido et al., 2018). The test was performed during 5 min. The total numbers of entries (four paws criteria) in open arms and in closed arms were recorded. The percentage of time spent in open and closed arms, and platform was calculated. In addition, the frequency of self-grooming (when mouse cleans, licks or scratches its body) as well as the presence of defecation in the holeboard and in the T-maze tests were also considered as anxiety-like behaviors (Archer, 1973; Sake and Graybiel, 2003).

2.6. Collection of peritoneal leukocytes

The peritoneal suspensions were obtained between 8 am and 10 am to minimize circadian variations in the immune system, by a procedure previously described, without animal sacrifice (Guayerbas et al., 2002a,b), which allowed monitoring the life span of the mice. Briefly, 3 ml of Hank's solution, adjusted to pH 7.4, were injected into the peritoneum, the abdomen was massaged and the peritoneal exudate cells were collected allowing the recovery of 90–95% of the injected volume. The peritoneal leukocytes, consisting of lymphocytes and macrophages, were counted in Neubauer chambers (Blau Brand, Wertheim, Germany). The suspensions were adjusted to a final concentration of 5×10^5 macrophages or lymphocytes per ml in Hank's solution or 10^6 leukocytes per ml in Hank's solution or complete medium (RPMI 1640 enriched with L-glutamine (PAA, Pasching, Austria) and supplemented with 10% heat-inactivated (56 °C, 30 min) fetal calf serum (PAA, Pasching, Austria) and gentamicin (100 mg/ml, PAA, Pasching, Austria) with or without phenol red, depending on the type of assay used. Macrophages and lymphocytes were identified by their morphology. The cellular viability was measured using the trypan-blue (Sigma-Aldrich, Madrid, Spain) exclusion test and in all cases was higher than 98%.

The immune functions and redox state parameters studied were performed using unfractionated peritoneal leukocytes, which better preserved the physiological environment surrounding the immune cells *in vivo* (Alonso-Fernández and De la Fuente, 2011).

2.7. Chemotaxis assay

Chemotaxis of peritoneal leukocytes was evaluated according to a slight modification of Boyden's method (Guayerbas et al., 2002a), consisting basically of the use of chambers with two compartments separated by a filter with a pore diameter of 3 μm (Millipore, Bedford, MA, USA). The chemotaxis index (CI) was determined by counting, in an optical microscope (100 ×), the total number of macrophages and lymphocytes on one third of the lower face of the filters.

2.8. Phagocytosis assay

Phagocytosis assay of inert particles (latex beads) was carried out following a method previously described (Guayerbas et al., 2002a). The number of particles ingested by 100 macrophages was counted using an optical microscope (100 ×) and expressed as phagocytic index (PI). The percentage of macrophages, which phagocyted at least one latex bead, was also determined and expressed as phagocytic efficiency (PE).

2.9. Natural killer assay

An enzymatic colorimetric assay was carried out to measure the cytolysis of tumor cells (murine lymphoma YAC-1 cells) (Cytotox 96 TM Promega, Boehringer Ingelheim, Germany) based on the determination of lactate dehydrogenase enzyme (LDH), as previously described (Guayerbas et al., 2002a). The results were expressed as percentage of lysis of tumor cells.

2.10. Lymphoproliferation assay

Following the method previously described (Guayerbas et al., 2002b), aliquots (200 μl) of peritoneal leukocytes (10^6 cells/ml complete medium) were seeded in 96 well flat-bottomed microtitre plates (Numc, Roskilde, Denmark) and 20 μl of concanavaline A (ConA 1 μg/ml, Sigma-Aldrich), 20 μl of lipopolysaccharide (LPS, *E. coli*, 055:B5 1 μg/ml, Sigma-Aldrich) or 20 μl of complete medium (spontaneous proliferation) were added per well. After 48 h of incubation at 37 °C in an atmosphere of 5% CO2, 0.5 μCi ^{3}H-thymidine (Du Pont, Boston, MA, USA) were added to each well. The cells were harvested in a semi-automatic microharvester 24 h later. Thymidine uptake was measured using a beta counter (LKB, Uppsala, Sweden). The results were expressed as ^{3}H-thymidine uptake (cpm).

2.11. Catalase activity assay

The activity of catalase (CAT) was determined following a previously described method (Alvarado et al., 2006). The enzymatic assay was followed using spectrophotometry for 80 s at 240 nm. The results were expressed as international units (U) of enzymatic activity per 10^6 cells.

2.12. Glutathione peroxidase activity assay

The glutathione peroxidase activity was determined according to

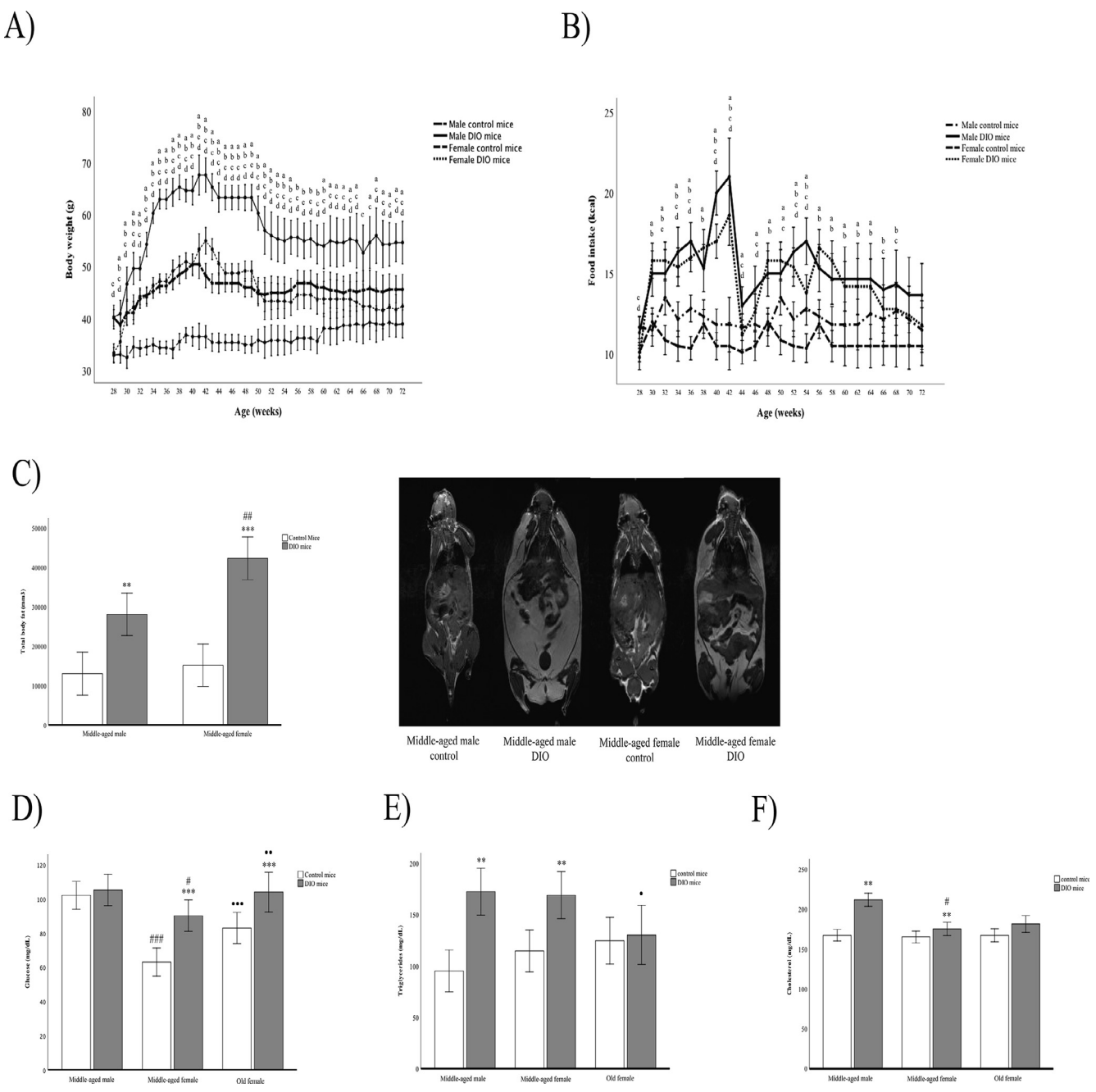


Fig. 1. (A) Body weight (g) of male and female control and diet-induced obese (DIO) mice during high-fat diet feeding (28–42 weeks of age) and aging (43–72 weeks old). (B) Food intake (kcal) of male and female control and DIO mice during high-fat diet feeding (28–42 weeks of age) and aging (43–72 weeks old). Significant differences: a: male control vs male DIO; b: female control vs female DIO; c: male control vs female control; d: male DIO vs female DIO. Data represent mean ± SEM (n = 3–10 animals for each group). (C) Total body fat mass (mm^3). Each column represents mean ± SEM (n = 4 animals for each group). $^{***}P < 0.001$; $^{**}P < 0.01$ with respect to the corresponding values of control mice. $^{\#\#}P < 0.01$ with respect to the corresponding values of middle-aged male DIO mice. (D) Glucose levels (mg/dL). $^{***}P < 0.001$ with respect to the corresponding values of control mice. $^{\#\#\#}P < 0.001$; $^{\#}P < 0.05$ with respect to the corresponding values of middle-aged male control mice and middle-aged male DIO mice. $^{\bullet\bullet\bullet}P < 0.001$; $^{\bullet\bullet}P < 0.01$ with respect to the corresponding values of middle-aged female control mice and middle-aged female DIO mice. Data represent median ± SEM (n = 5–10 animals for each group). (E) Triglyceride levels (mg/dL). $^{**}P < 0.01$ with respect to the corresponding values of control mice. Data represent median (IQR) (n = 5–10 animals for each group). (F) Total cholesterol levels (mg/dL). $^{**}P < 0.01$ with respect to the corresponding values of control mice. $^{\#}P < 0.05$ with respect to the corresponding values of middle-aged male DIO mice. Data represent median (IQR) (n = 5–10 animals for each group).

the method described by Lawrence and Burk (1976) with some modifications (Alvarado et al., 2006). The reaction was followed spectrophotometrically by the decrease of the absorbance at 340 nm. The results were expressed as milliunits (mU) of enzymatic activity per 10^6 cells.

2.13. GSH and GSSG concentrations assay

Both reduced (GSH) and oxidized (GSSG) concentrations of glutathione were measured using a fluorometric method, with some modifications (Garrido et al., 2018). Measurement of fluorescence was performed in a microplate reader using excitation at 350 nm and emission detection at 420 nm. The results were expressed as nmol/10^6

cells. The GSSH/GSH ratio was calculated for each sample.

2.14. Xanthine oxidase activity assay

Xanthine oxidase (XO) activity was measured by fluorescence using a commercial kit "Amplex Red Xanthine/Xanthine Oxidase Assay Kit" (Molecular Probes, Paisley, UK), as previously described (Vida et al., 2011). Measurement of fluorescence was performed in a microplate reader using excitation at 530 nm and emission detection at 595 nm. Data analysis was performed with xanthine standard curves at different concentrations, the results being expressed in international milliunits (mU) of enzymatic activity per 10^6 cells.

2.15. Lipid peroxidation (malondialdehyde) assay

The estimation of malondialdehyde (MDA) concentration in cells was evaluated using the commercial kit "MDA Assay Kit" (Biovision, Mountain View, CA, USA), which measures the reaction of MDA with thiobarbituric acid (TBA) and the MDA-TBA adduct formation, as previously described (Hunsche et al., 2018). The MDA-TBA adduct was measured using a spectrophotometer at 532 nm of absorbance. The results were expressed as nmol/10^6 cells.

2.16. Superoxide anion concentration assay

The superoxide anion concentration was evaluated assessing its capacity to reduce nitroblue tetrazolium (NBT, Sigma-Aldrich, Madrid, Spain), in an equimolecular reaction, following the method previously described (Guayerbas et al., 2002a). The reduced NBT was extracted with dioxin (Merck, Germany) and the absorbance of the supernatants was determined at 525 nm using a spectrophotometer. The data obtained were expressed as nmoles of NBT reduced per 10^6 leukocytes by extrapolating in a standard curve of NBT reduced with 1,4-dithoerythritol (Sigma-Aldrich, Madrid, Spain).

2.17. Statistical analysis

SPSS 25.0 (SPSS, Inc., Chicago, USA) was used for the statistical analysis of the results. The data were tested for normality of distribution (Kolmogorov-Smirnov test) and homogeneity of variance (Levene test). For non-normal distributions and non-homogeneous variances, the non-parametric Kruskal-Wallis and Friedman tests were used, and Mann-Whitney and Wilcoxon Signed Ranks to run pairwise comparisons, respectively. Two-way analysis of variance (ANOVA) with the factors being diet (standard and high-fat) and sex (male and female) was used to analyze results from middle-aged male and female mice. Two-way repeated ANOVA with the factors being diet (standard and high-fat diet) and age (middle-aged and old) was used to analyze results from old and middle-aged female mice. If significant effects were observed, post-hoc comparisons were performed as appropriate, with Bonferroni corrections for multiple comparisons. For comparison of qualitative data in behavioral tests, the chi-square was employed. Finally, a Kaplan-Meir survival analysis was conducted to verify the effects of obesity on the survival of male and female mice. The data were expressed as mean ± standard error of the mean (SEM), or median and interquartile range (IQR), depending upon the normality of data. P value < 0.05 was considered statistically significant.

3. Results

3.1. Body weight, total fat mass, food intake and biochemical parameters

After the period of 14 weeks on a high-fat diet, male and female diet-induced obese (DIO) mice displayed significantly higher body weight than their control counterparts, with males weighing more than females (Fig. 1A; Kruskal-Wallis test $X^2(3) = 31.059$, $P < 0.001$; significant differences: male control vs male DIO, $P < 0.001$; female control vs female DIO, $P < 0.001$; male control vs female control, $P < 0.001$; male DIO vs female DIO, $P < 0.001$, pairwise comparisons with Mann-Whitney). In addition, male and female DIO mice consumed significantly more kilocalories than their controls, with males consuming more kilocalories than females (Fig. 1B; Kruskal-Wallis test $X^2(3) = 29.631$, $P < 0.001$; significant differences: male control vs male DIO, $P < 0.001$; female control vs female DIO, $P < 0.001$; male control vs female control, $P = 0.010$; male DIO vs female DIO, $P = 0.008$, pairwise comparisons with Mann-Whitney). At old age (72 weeks old), male DIO mice continue to exhibit a higher body weight than their respective controls, with males weighing more than females (Fig. 1A; Kruskal-Wallis test $X^2(3) = 13.540$, $P = 0.004$; significant differences: male control vs male DIO, $P = 0.02$; male control vs female control, $P = 0.005$; male DIO vs female DIO, $P = 0.024$, pairwise comparisons with Mann-Whitney).

Male and female DIO mice showed higher total fat mass than the corresponding controls, with female DIO mice showing higher total body fat than male DIO mice (Fig. 1C; two-way ANOVA, significant interaction diet × sex $F(1,12) = 5.889$, $P = 0.032$, main effect of diet $F(1,12) = 71.798$, $P < 0.001$, main effect of sex $F(1,12) = 10.797$, $P = 0.007$; significant differences: male control vs male DIO, $P = 0.001$; female control vs female DIO, $P < 0.001$; male DIO vs female DIO, $P = 0.002$, post-hoc comparisons with Bonferroni corrections).

Female DIO mice showed higher values of glucose than their respective non-DIO counterparts. In addition, males displayed significantly higher levels of glucose than females (Fig. 1D; two-way ANOVA, significant interaction diet × sex $F(1,32) = 6.187$, $P = 0.018$, main effect of diet $F(1,32) = 9.716$, $P = 0.004$, main effect of sex $F(1,32) = 31.178$, $P < 0.001$; significant differences: female control vs female DIO, $P < 0.001$; male control vs female control, $P < 0.001$; male DIO vs female DIO, $P = 0.046$, post-hoc comparisons with Bonferroni corrections). The levels of triglycerides were significantly higher in male and female DIO mice in comparison with their respective non-DIO controls (Fig. 1E; Kruskal-Wallis test $X^2(3) = 25.135$, $P < 0.001$; significant differences: male control vs male DIO, $P = 0.001$; female control vs female DIO, $P = 0.003$, pairwise comparisons with Mann-Whitney). In addition, the levels of total cholesterol were higher in male and female DIO mice than in their respective non-DIO mice, with male DIO mice showing higher cholesterol levels than female DIO mice (Fig. 1F; Kruskal-Wallis test $X^2(3) = 20.255$, $P < 0.001$; significant differences: male control vs male DIO, $P = 0.001$; female control vs female DIO, $P = 0.001$; male DIO vs female DIO, $P = 0.013$, pairwise comparisons with Mann-Whitney).

At old age, when females reached 72 weeks of age, they exhibited higher levels of glucose than middle-aged females (Fig. 1D; two-way repeated ANOVA measures, significant main effect of diet $F(1,11) = 43.099$, $P < 0.001$, main effect of age $F(1,11) = 31.385$, $P < 0.001$; significant differences: old female control vs old female DIO, $P < 0.001$; middle-aged female control vs old female control, $P < 0.001$; middle-aged female DIO vs old female DIO, $P = 0.007$, post-hoc comparisons with Bonferroni corrections). In addition, old female DIO mice showed lower levels of triglycerides than middle-aged female DIO mice (Fig. 1E; Friedman test, $X^2(3) = 9.000$, $P = 0.029$; significant differences: middle-aged female DIO vs old female DIO, $P = 0.043$, pairwise comparisons with Wilcoxon Signed Ranks).

3.2. Behavioral tests

3.2.1. Reflexes

No significant differences were found between middle-aged male and female DIO mice and their respective non-DIO counterparts in reflexes (male control: 100%; male DIO: 100%; female control: 100%; female DIO: 100%).

Table 1
Behavioral tests in middle-aged male and female control and middle-aged male and female DIO (diet-induced obese) mice.

	Middle-aged male		Middle-aged female	
	Control	DIO	Control	DIO
Motor coordination and equilibrium (Wood rod test)				
% of mice falling off	0	10^{**}	0	$0^{\#\#}$
% of mice that cover at least 1 segment	100	80^{***}	100	$100^{\#\#\#}$
% of mice that complete the test	100	80^{***}	100	$100^{\#\#\#}$
Time to leave first segment (s)	4 ± 1	5 ± 1	2 ± 0	4 ± 1
Muscular vigor (Tightrope test)				
% of mice falling off	33	75^{***}	25	$40^{*\#\#\#}$
% of mice that complete test	67	25^{***}	75	$60^{*\#\#\#}$
% of mice that show maximum traction capacity	80	50^{***}	$100^{\#\#\#}$	$80^{***\#\#\#}$
Vertical exploratory activity				
Total number of rearings (Corner test)	8 ± 1	$6 \pm 1^{*}$	8 ± 1	$5 \pm 1^{*}$
Total number of rearings (Holeboard test)	24 ± 2	$15 \pm 2^{***}$	$34 \pm 2^{\#\#\#}$	$27 \pm 2^{**\#\#\#}$
Horizontal exploratory activity				
Total number of corners visited (Corner test)	11 ± 1	9 ± 1	11 ± 1	$8 \pm 1^{*}$
Total number of line crossings (Holeboard test)	354 ± 15	$275 \pm 16^{**}$	296 ± 16	301 ± 15
Number of line crossings in the peripheral area (Holeboard test)	231 ± 11	$188 \pm 13^{*}$	$172 \pm 13^{\#\#}$	198 ± 11
Number of line crossings in the central area (Holeboard test)	123 ± 6	$87 \pm 7^{***}$	125 ± 7	$104 \pm 6^{*}$
Time to complete the test (s) (T-maze test)	17(3)	$56(52)^{***}$	21(6)	$23(6)^{*\#\#}$
Anxiety-like behavior				
Total number of entries in open arms (Elevated plus maze test)	8 ± 1	6 ± 2	13 ± 2	9 ± 1
% of time in open arms (Elevated plus maze test)	36 ± 2	$16 \pm 2^{***}$	39 ± 2	$31 \pm 2^{*\#\#\#}$
Total number of entries in closed arms (Elevated plus maze test)	8 ± 2	14 ± 2	11 ± 2	11 ± 2
% of time in closed arms (Elevated plus maze test)	37 ± 2	$55 \pm 2^{***}$	37 ± 2	$43 \pm 2^{\#\#\#}$
% of time in central platform (Elevated plus maze test)	25 ± 3	30 ± 3	26 ± 3	27 ± 3
Total number of self-grooming (T-maze test)	0 ± 0	0 ± 0	0 ± 0	1 ± 0
Time of self-grooming (s) (Holeboard test)	2(4)	$15(9)^{***}$	2(8)	$7(6)^{*\#}$
Total number of fecal bolei (Holeboard test)	1 ± 0	$4 \pm 0^{***}$	2 ± 0	$4 \pm 0^{***}$
% fecal bolei presence (T-maze test)	0	40^{***}	0	$20^{***\#\#}$

Each value represents the mean ± SEM or median (IQR) (n = 8–10 animals for each group). $^{***}P < 0.001$; $^{**}P < 0.01$; $^{*}P < 0.05$ with respect to the values of the corresponding control mice. $^{\#\#\#}P < 0.001$; $^{\#\#}P < 0.01$; $^{\#}P < 0.05$ with respect to the values of middle-aged male control mice and middle-aged male DIO mice.

3.2.2. Motor coordination and equilibrium

The abilities of motor coordination and equilibrium that were evaluated, using the wood rod test, are shown in Table 1. The percentage of falls from the wood rod was significantly higher in male DIO mice than in their respective non-DIO controls (Table 1; Chi square test, $X^2(1) = 8.526$, $P = 0.004$). In addition, male DIO mice displayed significantly lower percentages of test completion and the covering of one segment in the wood rod test than male non-DIO mice (Table 1; Chi square test, $X^2(1) = 20.056$, $P < 0.001$). No differences were observed in these parameters between female DIO mice and their respective non-DIO controls (Table 1). In the wood rod test, male DIO mice showed a worse performance in the percentage of falls (Table 1; Chi square test, $X^2(1) = 8.526$, $P = 0.004$), on test completion (Table 1; Chi square test, $X^2(1) = 20.056$, $P < 0.001$) and the covering of one segment (Table 1; Chi square test, $X^2(1) = 20.056$, $P < 0.001$) than female DIO mice.

3.2.3. Muscular vigor

The muscular vigor characteristics of mice, which were evaluated by the tightrope test, are shown in Table 1. Male and female DIO mice showed significantly higher percentage of falls from the rope than their respective non-DIO controls (Table 1; Chi square test, $X^2(1) = 33.837$, $P < 0.001$) for male and (Table 1; Chi square test, $X^2(1) = 4.467$, $P = 0.035$) for female. In addition, the percentage of the test completed was significantly lower in male and female DIO mice in comparison with their respective controls (Table 1; Chi square test, $X^2(1) = 33.837$, $P < 0.001$) for male and (Table 1; Chi square test, $X^2(1) = 4.467$, $P = 0.035$) for female. The percentage of mice that used their forelimbs, hindlimbs and tail to remain hanging and consequently showed maximum traction capacity, was significantly lower in male and female DIO mice in comparison with their respective controls (Table 1; Chi square test, $X^2(1) = 18.483$, $P < 0.001$) for male and (Table 1; Chi square test, $X^2(1) = 20.056$, $P < 0.001$) for female. Male DIO mice showed a worse response in the percentage of falls (Table 1; Chi square test, $X^2(1) = 23.652$, $P < 0.001$), test completed (Table 1; Chi square test, $X^2(1) = 23.652$, $P < 0.001$), and traction capacity (Table 1; Chi square test, $X^2(1) = 18.484$, $P < 0.001$) than female DIO mice.

3.2.4. Vertical exploratory activity

The vertical exploratory activity of mice, which was evaluated by the performance of rearing in the corner and holeboard tests, is shown in Table 1. The total number of rearings in the corner test was significantly lower in male and female DIO mice than their respective non-DIO controls (Table 1; two-way ANOVA, significant main effect of diet $F(1,32) = 11.083$, $P = 0.002$; significant differences: male control vs male DIO, $P = 0.027$; female control vs female DIO, $P = 0.023$, post-hoc comparisons with Bonferroni corrections). In addition, male and female DIO mice performed significantly lower number of rearings in the holeboard test than their respective controls, with males showing a lower total of rearings than females (Table 1; two-way ANOVA, significant main effect of diet $F(1,32) = 25.176$, $P < 0.001$, main effect of sex $F(1,32) = 45.773$, $P < 0.001$; significant differences: male control vs male DIO, $P < 0.001$; female control vs female DIO, $P = 0.005$; male control vs female control, $P < 0.001$; male DIO vs female DIO, $P < 0.001$, post-hoc comparisons with Bonferroni corrections).

3.2.5. Horizontal exploratory activity

The horizontal exploratory activity was evaluated by the ambulation of mice in the corner, holeboard and T-maze tests (Table 1). Female DIO mice visited lower number of corners in the corner test than their respective controls (Table 1; two-way ANOVA, significant main effect of diet $F(1,32) = 4.861$, $P = 0.035$; significant differences: female control vs female DIO, $P = 0.049$, post-hoc comparisons with Bonferroni corrections). Male DIO mice performed lower number of total line crossings than their respective controls (Table 1; two-way ANOVA,

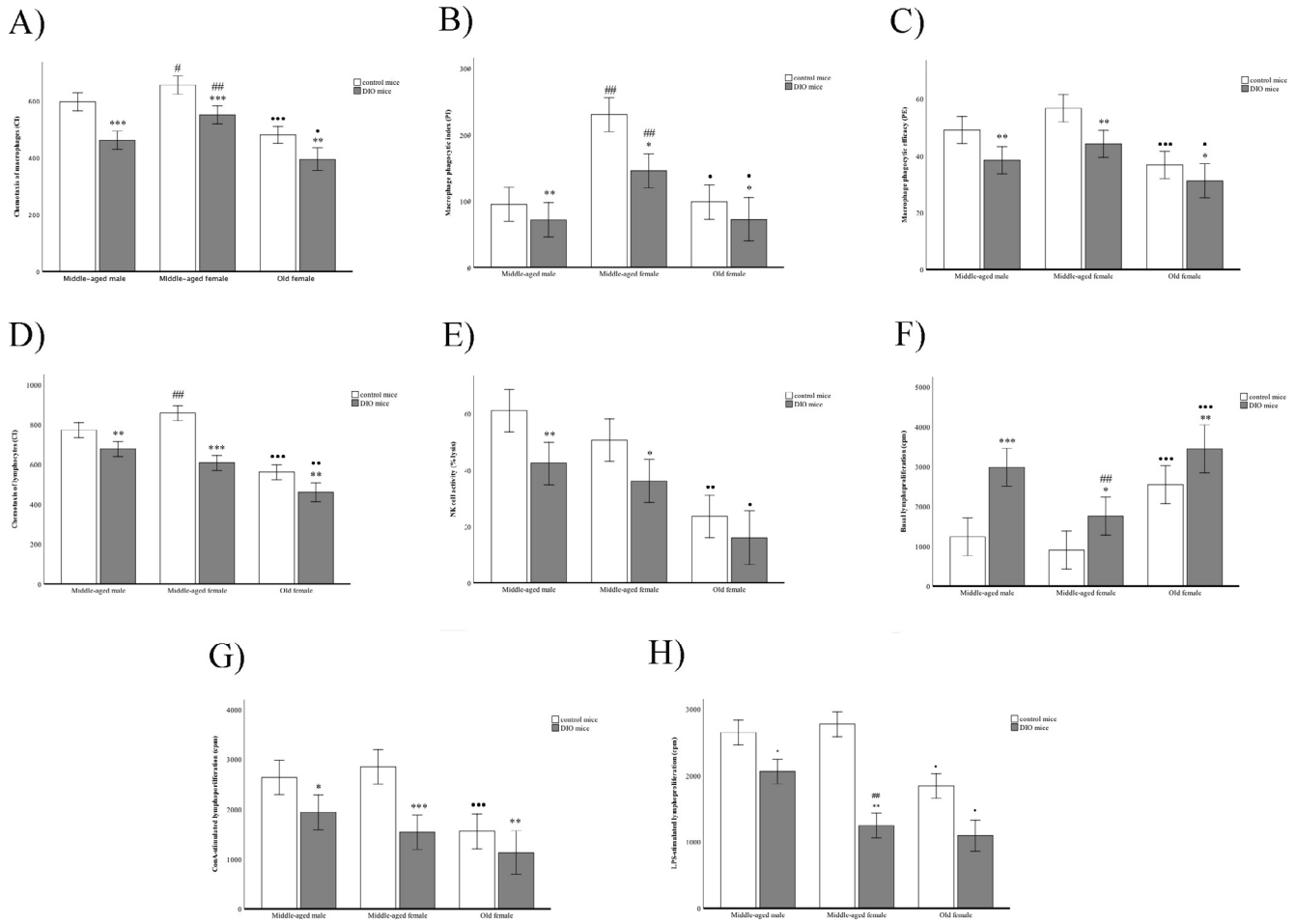


Fig. 2. Immune cell functions. (A) Macrophage chemotaxis index (CI, number of macrophages). (B) Macrophage phagocytic index (PI, number latex beads/100 macrophages). (C) Macrophage phagocytic efficacy (PE, number of phagocyting macrophages/100 macrophages). (D) Lymphocyte chemotaxis index (CI, number of lymphocytes). (E) Natural killer cell activity (% lysis). (F) Basal lymphoproliferation (cpm). (G) Lymphoproliferative response to concanalvaline A (ConA) (cpm). (H) Lymphoproliferative response to lipopolysaccharide (LPS) (cpm). Data represent mean ± SEM or median (IQR) (n = 5–10 animals for each group). $^{***}P < 0.001$; $^{**}P < 0.01$; $^{*}P < 0.05$ with respect to the corresponding values of control mice. $^{\#\#}P < 0.01$; $^{\#}P < 0.05$ with respect to the corresponding values of middle-aged male control mice and middle-aged male DIO mice. $^{\cdot\cdot\cdot}P < 0.001$; $^{\cdot\cdot}P < 0.01$; $^{\cdot}P < 0.05$ with respect to the values of middle-aged female control and DIO mice and middle-aged female DIO mice.

significant main effect of diet F(1,32) = 5.563, $P = 0.025$; significant differences: male control vs male DIO, $P = 0.001$, post-hoc comparisons with Bonferroni corrections). Male DIO mice displayed significantly lower peripheral ambulation (which was measured by the number of line crossings in the peripheral area of the holeboard) than their respective controls. Also, male controls showed significantly higher peripheral ambulation than female controls (Table 1; two-way ANOVA, significant interaction diet × sex F(1,32) = 8.341, $P = 0.007$, main effect of sex F(1,32) = 4.460, $P = 0.043$; significant differences: male control vs male DIO, $P = 0.016$; male control vs female control, $P = 0.001$, post-hoc comparisons with Bonferroni corrections). In addition, male and female DIO mice showed a significant lower central ambulation (which were measured by the number of line crossings in the central area of the holeboard) than their respective non-DIO controls (Table 1; two-way ANOVA, significant main effect of diet F(1,32) = 20.303, $P < 0.001$; significant differences: male control vs male DIO, $P < 0.001$; female control vs female DIO, $P = 0.023$, post-hoc comparisons with Bonferroni corrections).

Male and female DIO mice took a longer time to complete the exploration of the three arms of the T-maze test than their non-DIO counterparts, with male DIO mice showing a longer time to complete the test than female DIO mice (Table 1; Kruskal-Wallis test $X^2(3) = 23.553$, $P < 0.001$; significant differences: male control vs male DIO, $P < 0.001$; female control vs female DIO, $P = 0.044$; male DIO vs female DIO, $P = 0.001$, pairwise comparisons with Mann-Whitney).

3.2.6. Goal-directed exploratory behavior

The goal-directed exploratory behavior, which was evaluated by the percentage and total number of mice that performed head-dips in the holeboard test, was not significantly different between the studied groups for percentage of head-dips (male control: 100%; male DIO: 100%; female control: 100%; female DIO: 100%), and for total number of head-dips (male control: 12 ± 4; male DIO: 10 ± 5; female control: 13 ± 5; female DIO: 10 ± 2).

3.2.7. Anxiety-like behavior

The anxiety-like behavior, which was assessed by the elevated plus maze test, is shown in Table 1. Male and female DIO mice exhibited a lower percentage of time in open arms than their respective non-DIO controls, with male DIO mice showing a lower percentage of time than female DIO mice (Table 1; two-way ANOVA, significant interaction diet × sex F(1,32) = 6.820, $P = 0.014$, main effect of diet F(1,32) = 36.992, $P < 0.001$, main effect of sex F(1,32) = 17.599, $P < 0.001$; significant differences: male control vs male DIO, $P < 0.001$; female control vs female DIO, $P = 0.02$; male DIO vs

female DIO, $P < 0.001$, post-hoc comparisons with Bonferroni corrections). Moreover, the percentage of time in closed arms was higher in male DIO mice in comparison with their respective controls and female counterparts (Table 1; two-way ANOVA, significant interaction diet × sex $F(1,32) = 8.932$, $P = 0.005$, main effect of diet $F(1,32) = 30.330$, $P < 0.001$, main effect of sex $F(1,32) = 7.884$, $P = 0.008$; significant differences: male control vs male DIO, $P < 0.001$; male DIO vs female DIO, $P < 0.001$, post-hoc comparisons with Bonferroni corrections). In addition, the time (in seconds) spent by mice performing self-grooming in the holeboard test was significantly higher in male and female DIO mice than in their respective controls (Table 1; Kruskal-Wallis test $X^2(3) = 21.087$, $P < 0.001$; significant differences: male control vs male DIO, $P < 0.001$; female control vs female DIO, $P = 0.027$; male DIO vs female DIO, $P = 0.027$, pairwise comparisons with Mann-Whitney).

Male and female DIO mice also showed a higher percentage of fecal bolei presence (Table 1; Chi square test, $X^2(1) = 47.531$, $P < 0.001$) for male and ($X^2(1) = 20.056$, $P < 0.001$) for female, with male DIO mice having a higher percentage of fecal bolei presence than female DIO mice (Table 1; Chi square test, $X^2(1) = 8.595$, $P = 0.003$). In addition, the total number of fecal bolei was higher in male and female DIO mice than their respective non-DIO controls (Table 1; Kruskal-Wallis test $X^2(3) = 23.908$, $P < 0.001$; significant differences: male control vs male DIO, $P < 0.001$; female control vs female DIO, $P < 0.001$, pairwise comparisons with Mann-Whitney).

3.3. Peritoneal leukocyte functions

The peritoneal macrophage functions are shown in Fig. 2. The migration of peritoneal macrophages in response to a chemotactic gradient (formylated peptide), which mimics the migration of immune cells towards the infection site, was significantly lower in male and female DIO mice compared with their respective non-DIO controls. Males displayed significantly lower chemotactic index (CI) than females (Fig. 2A; two-way ANOVA, significant main effect of diet $F(1,28) = 51.573$, $P < 0.001$, significant main effect of sex $F(1,28) = 19.477$, $P < 0.001$; significant differences: male control vs male DIO, $P < 0.001$; female control vs female DIO, $P < 0.001$; male control vs female control, $P = 0.019$; male DIO vs female DIO, $P = 0.001$, post-hoc comparisons with Bonferroni corrections). In addition, the number of latex beads ingested by macrophages (the phagocytic index) was lower in male and female DIO mice with respect to the corresponding controls, with males displaying a lower phagocytic index than females (Fig. 2B; Kruskal-Wallis test $X^2(3) = 26.087$, $P < 0.001$; significant differences: male control vs male DIO, $P = 0.002$; female control vs female DIO, $P = 0.016$, male control vs female control, $P = 0.001$; male DIO mice vs female DIO mice, $P = 0.001$, pairwise comparisons with Mann-Whitney). The number of macrophages with phagocytic ability (measured by phagocytic efficacy) were also lower in male and female DIO mice with respect to the corresponding controls (Fig. 2C; two-way ANOVA, significant main effect of diet $F(1,28) = 19.211$, $P < 0.001$; significant differences: male control vs male DIO, $P = 0.008$; female control vs female DIO, $P = 0.002$, post-hoc comparisons with Bonferroni corrections).

With aging, the chemotactic index (CI) was significantly lower in old female DIO mice than in their corresponding non-DIO mice, with old females displaying a lower CI than middle-aged females (Fig. 2A; two-way repeated measures ANOVA, significant main effect of diet $F(1,11) = 30.551$, $P < 0.001$, main effect of age $F(1,11) = 41.213$, $P < 0.001$; significant differences: old female control vs old female DIO, $P = 0.002$; middle-aged female control vs old female control, $P < 0.001$; middle-aged female DIO vs old female DIO, $P = 0.010$, post-hoc comparisons with Bonferroni corrections). In addition, the phagocytic index (PI) was significantly lower in old female DIO mice than in non-DIO mice, with old females displaying a lower PI than middle-aged females (Fig. 1B; Friedman test, $X^2(3) = 13.653$, $P = 0.003$; significant differences: old female control mice vs old female DIO mice, $P = 0.012$, middle-aged female control vs old female control, $P = 0.012$, middle-aged female DIO vs old female DIO, $P = 0.043$, pairwise comparisons with Wilcoxon Signed Ranks). The phagocytic efficacy (PE) was significantly lower in old female DIO mice than in their corresponding controls, with old females displaying a lower PE than middle-aged females (Fig. 2C; two-way repeated measures ANOVA, significant main effect of diet $F(1,11) = 12.892$, $P = 0.004$, main effect of age $F(1,11) = 30.985$, $P < 0.001$; significant differences: old female control vs old female DIO, $P = 0.032$; middle-aged female mice vs old female control, $P < 0.001$; middle-aged female DIO vs old female DIO, $P = 0.015$, post-hoc comparisons with Bonferroni corrections).

The chemotactic index (CI) of peritoneal lymphocytes was significantly lower in male and female DIO mice in comparison with their non-DIO counterparts. Male control mice displayed lower CI than female control mice (Fig. 2D; two-way ANOVA, significant interaction diet × sex $F(1,28) = 17.467$, $P < 0.001$, main effect of diet $F(1,28) = 86.028$, $P < 0.001$; significant differences: male control vs male DIO, $P = 0.001$; female control vs female DIO, $P < 0.001$; male control vs female control, $P = 0.003$, post-hoc comparisons with Bonferroni corrections).

The NK activity was significantly lower in male and female DIO mice in comparison with their non-DIO controls (Fig. 2E; two-way ANOVA, significant interaction diet × sex $F(1,28) = 17.467$, $P < 0.001$, main effect of diet $F(1,28) = 16.420$, $P < 0.001$; significant differences: male control vs male DIO, $P = 0.003$; female control vs female DIO, $P = 0.019$, post-hoc comparisons with Bonferroni corrections).

The basal proliferative capacity of peritoneal lymphocytes as well as in response to T-cell mitogen (ConA) and to B-cell mitogen (LPS) are shown in Fig. 2F, G and H, respectively. Male and female DIO mice displayed significantly higher basal lymphoproliferation in comparison with non-DIO controls, with male DIO mice showing higher basal lymphoproliferation than female DIO mice (Fig. 2F; two-way ANOVA, significant main effect of diet $F(1,28) = 24.967$, $P < 0.001$; main effect of sex $F(1,28) = 8.982$, $P = 0.006$; significant differences: male control vs male DIO, $P < 0.001$; female control vs female DIO, $P = 0.028$; male DIO vs female DIO, $P = 0.002$, post-hoc comparisons with Bonferroni corrections). However, in response to the mitogen ConA, the proliferation of lymphocytes was lower in male and female DIO mice in comparison with their controls (Fig. 2G; two-way ANOVA, significant main effect of diet $F(1,28) = 25.513$, $P < 0.001$; significant differences: male control vs male DIO, $P = 0.019$; female control vs female DIO, $P < 0.001$, post-hoc comparisons with Bonferroni corrections). Male and female DIO mice also showed lower LPS-stimulated proliferation in comparison with their non-DIO mice, with male DIO mice showing higher LPS-stimulated proliferation than female DIO mice (Fig. 2H; Kruskal-Wallis test $X^2(3) = 23.172$, $P < 0.001$; significant differences: male control vs male DIO, $P = 0.021$; female control vs female DIO, $P = 0.001$; male DIO vs female DIO, $P = 0.001$, pairwise comparisons with Mann-Whitney).

With aging, the chemotactic index (CI) of peritoneal lymphocytes in old female DIO mice was significantly lower when compared with their corresponding non-DIO mice, with old females displaying lower CI than middle-aged females (Fig. 2D; two-way repeated measures ANOVA, significant interaction diet × age $F(1,11) = 13.264$, $P = 0.004$, main effect of diet $F(1,11) = 72.556$, $P < 0.001$, main effect of age $F(1,11) = 92.563$, $P < 0.001$; significant differences: old female control vs old female DIO, $P = 0.005$; middle-aged female control vs old female control, $P < 0.001$; middle-aged female DIO vs old female DIO, $P = 0.003$, post-hoc comparisons with Bonferroni corrections). Old females showed significantly lower anti-tumor NK activity than middle-aged females (Fig. 2E; two-way repeated measures ANOVA, significant main effect of age $F(1,11) = 27.653$, $P < 0.001$; significant differences: middle-aged female control vs old female control, $P = 0.001$;

middle-aged female DIO vs old female DIO, $P = 0.011$, post-hoc comparisons with Bonferroni corrections).

The basal proliferation of lymphocytes in old female DIO mice was higher than in their respective controls, with old females displaying higher basal lymphoproliferation than middle-aged females (Fig. 2F; two-way repeated measures ANOVA, significant main effect of diet F(1,11) = 24.379, $P < 0.001$, main effect of age F(1,11) = 150.177, $P < 0.001$; significant differences: old female control vs old female DIO, $P = 0.006$; middle-aged female control vs old female control, $P < 0.001$; middle-aged female DIO vs old female DIO, $P < 0.001$, post-hoc comparisons with Bonferroni corrections). Nevertheless, old female DIO mice exhibited significantly lower proliferation of lymphocytes in response to ConA mitogen-stimulated condition (Fig. 2G; two-way repeated measures ANOVA, significant main effect of diet F(1,11) = 46.743, $P < 0.001$, main effect of age F(1,11) = 22.975, $P = 0.001$; significant differences: old female control vs old female DIO, $P = 0.001$; middle-aged female control vs old female control, $P < 0.001$, post-hoc comparisons with Bonferroni corrections). In addition, old females displayed significantly lower LSP-stimulated lymphoproliferation than middle-aged females (Fig. 2H; Friedman test, $X^2(3) = 13.776$, $P = 0.003$; significant differences: middle-aged female control vs old female control, $P = 0.012$; middle-aged female DIO vs old female DIO, $P = 0.043$, pairwise comparisons with Wilcoxon Signed Ranks).

3.4. Peritoneal leukocyte oxidative stress parameters

Fig. 3 shows the results of enzymatic and non-enzymatic antioxidants, such as catalase (CAT) and glutathione peroxidase (GPx) activities as well as reduced glutathione (GSH) concentrations, respectively. The CAT activity was lower in leukocytes of male and female DIO mice in comparison with their corresponding non-DIO controls (Fig. 3A; two-way ANOVA, significant main effect of diet F(1,28) = 37.361, $P < 0.001$; significant differences: male control vs male DIO, $P < 0.001$; female control vs female DIO, $P < 0.001$, post-hoc comparisons with Bonferroni corrections). The GPx activity in leukocytes of male and female DIO mice was higher in comparison with their respective controls, with male control mice showing lower GPx activity than female control mice (Fig. 3B; two-way ANOVA, significant main effect of diet F(1,28) = 33.633, $P < 0.001$, main effect of sex F(1,28) = 17.618, $P < 0.001$; significant differences: male control vs male DIO, $P < 0.001$; female control vs female DIO, $P = 0.003$, male control vs female control, $P = 0.001$, male DIO vs female DIO, $P = 0.043$, post-hoc comparisons with Bonferroni corrections). The GSH concentrations were lower in peritoneal leukocytes of male and female DIO mice in comparison with their respective non-DIO controls (Fig. 3C; two-way ANOVA, significant main effect of diet F(1,28) = 27.421, $P < 0.001$; significant differences: male control vs male DIO, $P = 0.004$; female control vs female DIO, $P < 0.001$, post-hoc comparisons with Bonferroni corrections).

With aging, old female control mice displayed significantly lower CAT activity than middle-aged female control mice (Fig. 3A; two-way repeated measures ANOVA, significant main effect of age F(1,11) = 22.686, $P = 0.001$; significant differences: middle-aged female control vs old female control, $P < 0.001$, post-hoc comparisons with Bonferroni corrections). Old females also exhibited lower GPx activity than middle-aged females (Fig. 3B; two-way repeated measures ANOVA, significant main effect of age F(1,11) = 80.339, $P < 0.001$; significant differences: middle-aged female control vs old female control, $P < 0.001$; middle-aged female DIO vs old female DIO, $P < 0.001$, post-hoc comparisons with Bonferroni corrections). Regarding the GSH concentrations, old female DIO mice displayed lower values than those of old female non-DIO mice, with old females showing lower concentrations of GSH than middle-aged females (Fig. 3C; two-way repeated measures ANOVA, significant main effect of diet F(1,11) = 9.116, $P = 0.012$, main effect of age F(1,11) = 21.591, $P = 0.001$; significant differences: old female control vs old female DIO, $P = 0.005$, middle-aged female control vs old female control, $P = 0.001$, middle-aged female DIO vs old female DIO, $P = 0.033$, post-hoc comparisons with Bonferroni corrections).

Fig. 4 shows the values of enzymatic and non-enzymatic oxidants, including the activity of xanthine oxidase, the concentrations of MDA and superoxide anion as well as the GSSG/GSH ratios. The activity of xanthine oxidase, which is associated with the production of free radicals, was significantly higher in male and female DIO mice in comparison with their corresponding non-DIO controls, with males showing higher XO activity than females (Fig. 4A; two-way ANOVA, significant main effect of diet F(1,28) = 58.360, $P < 0.001$, main effect of sex F(1,28) = 34.629, $P < 0.001$; significant differences: male control vs male DIO, $P < 0.001$; female control vs female DIO, $P < 0.001$; male control vs female control, $P < 0.001$; male DIO vs female DIO, $P < 0.001$, post-hoc comparisons with Bonferroni corrections). The MDA concentrations, which are an indicator of lipid oxidation and oxidative damage in cells, were significantly higher in male and female DIO mice with respect to their non-DIO controls, with male DIO mice showing higher MDA concentrations than female DIO mice (Fig. 4B; two-way ANOVA, significant interaction diet × sex F(1,28) = 8.633, $P = 0.007$, main effect of diet F(1,28) = 61.532, $P < 0.001$, main effect of sex F(1,28) = 8.475, $P < 0.001$; significant differences: male control vs male DIO, $P < 0.001$; female control vs female DIO, $P = 0.002$; male DIO vs female DIO, $P < 0.001$, post-hoc comparisons with Bonferroni corrections). The concentrations of superoxide anion were significantly higher in male and female DIO mice with respect to their non-DIO controls (Fig. 4C; two-way ANOVA, significant main effect of diet F(1,28) = 20.017, $P < 0.001$; significant differences: male control vs male DIO, $P = 0.001$; female control vs female DIO, $P = 0.017$, post-hoc comparisons with Bonferroni corrections). The GSSG/GSH ratios, an indicator of oxidative stress, were significantly higher in male and female DIO mice than in their respective non-DIO controls (Fig. 4D; two-way ANOVA, significant main effect of diet F

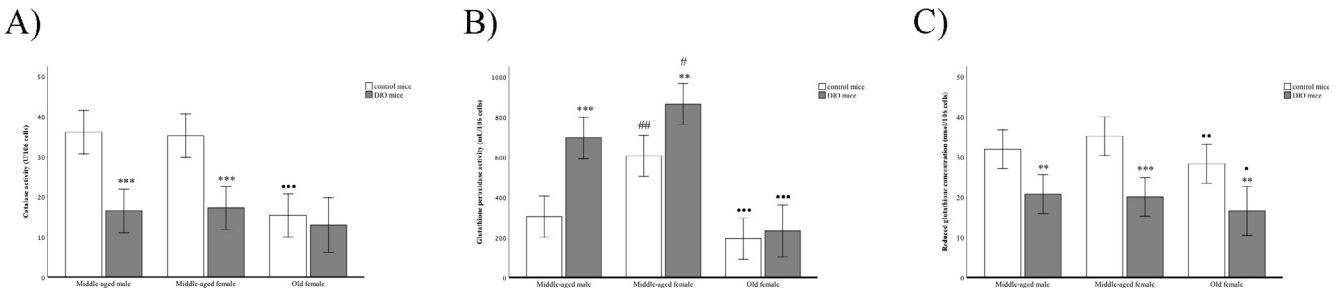


Fig. 3. Antioxidant parameters. (A) Catalase activity ($U/10^6$ cells). (B) Glutathione peroxidase activity ($mU/10^6$ cells). (C) Reduced glutathione concentration (GSH) ($nmol/10^6$ cells). Data represent mean ± SEM (n = 5–10 animals for each group). $^{***}P < 0.001$; $^{**}P < 0.01$ with respect to the corresponding values of control mice. $^{\#\#}P < 0.01$ with respect to the corresponding values of middle-aged male control mice and middle-aged male DIO mice. $^{\dddot{}}P < 0.001$; $^{\ddot{}}P < 0.01$; $^{\dot{}}P < 0.05$ with respect to the values of middle-aged female control mice and middle-aged female DIO mice.

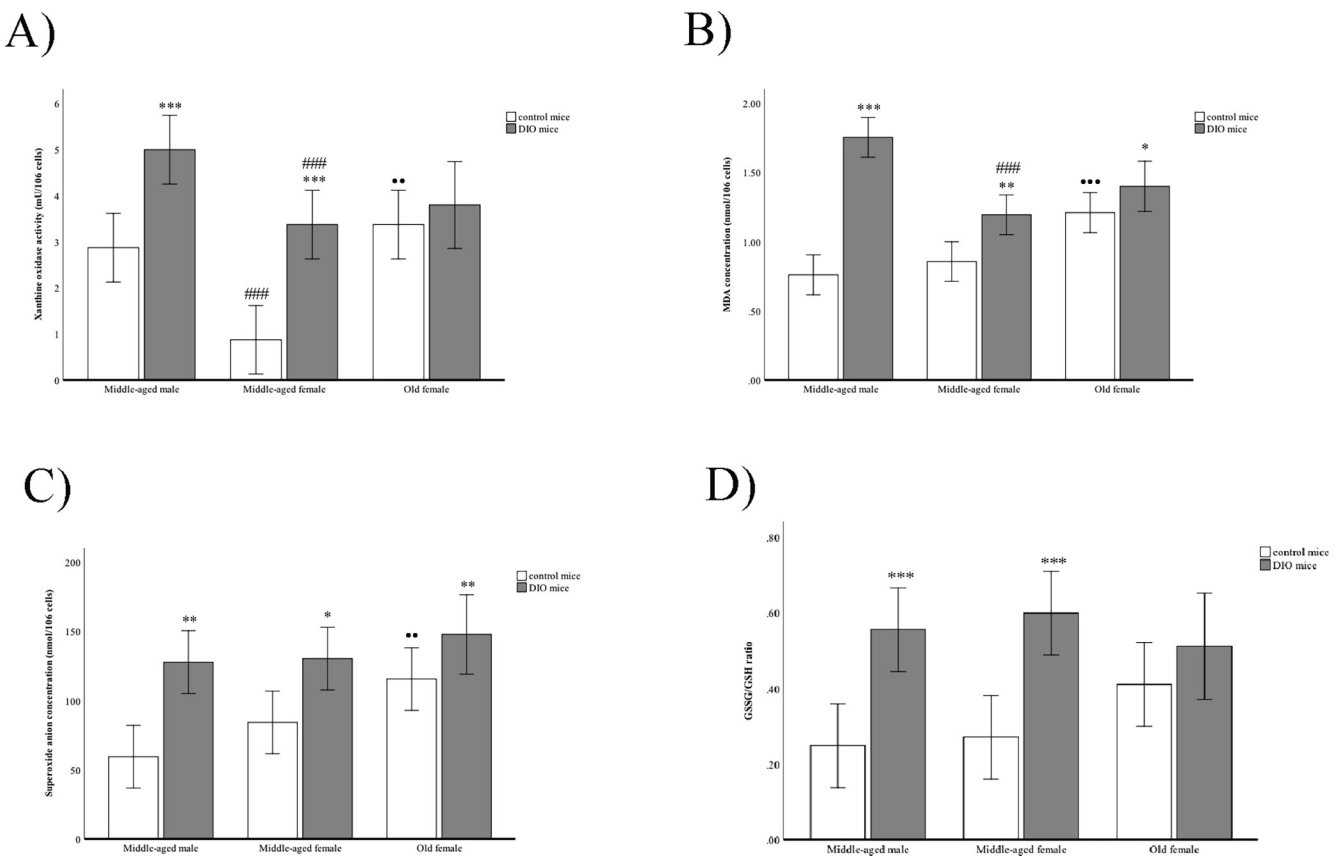


Fig. 4. Oxidative parameters. (A) Xanthine oxidase activity (mU/10^6 cells). (B) MDA concentration (nmol/10^6 cells). (C) Superoxide anion concentration (nmol/10^6 cells). (D) Oxidized glutathione (GSSG)/Reduced glutathione (GSH) ratio. Data represent mean ± SEM (n = 5–10 animals for each group). $^{***}P < 0.001$; $^{**}P < 0.01$; $^{*}P < 0.05$ with respect to the corresponding values of control mice. $^{\#\#\#}P < 0.001$ with respect to the corresponding values of middle-aged male control mice and middle-aged male DIO mice. $^{\cdot\cdot\cdot}P < 0.001$; $^{\cdot\cdot}P < 0.01$ with respect to the values of middle-aged female control mice and middle-aged female DIO mice.

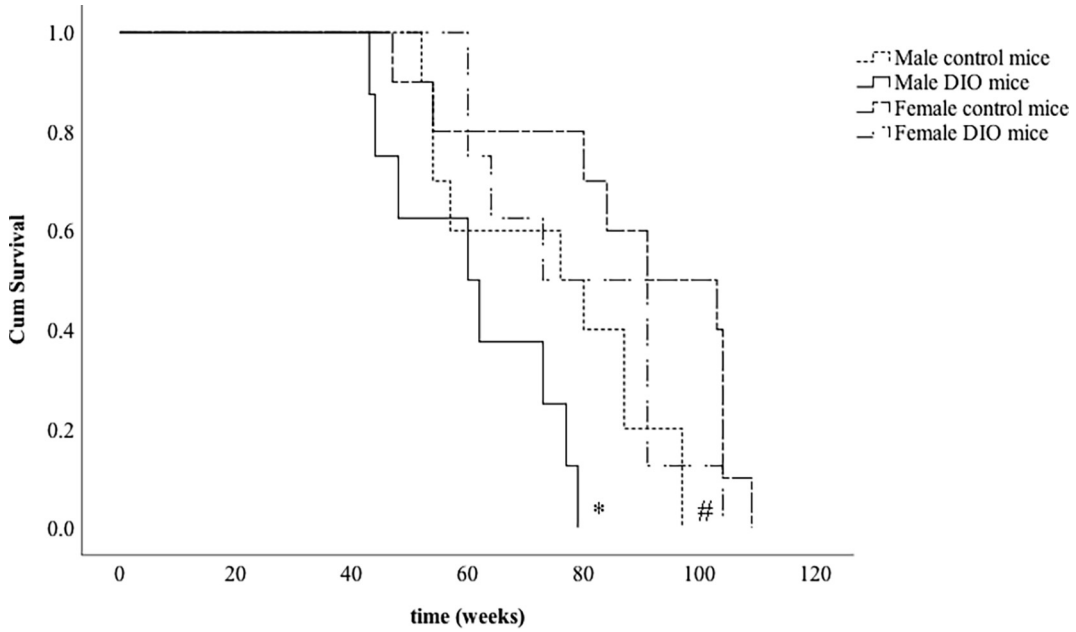


Fig. 5. Mortality records from male and female control mice and from male and female DIO (diet-induced obese) mice. $^{*}P < 0.05$ with respect to the corresponding values of middle-aged control mice. $^{\#}P < 0.05$ with respect to the corresponding values of middle-aged male DIO mice.

(1,28) = 75.625, $P < 0.001$; significant differences: male control vs male DIO, $P < 0.001$; female control vs female DIO, $P < 0.001$, post-hoc comparisons with Bonferroni corrections).

With aging, the xanthine oxidase activity in old female controls was significantly higher than in middle-aged female controls (Fig. 4A; two-way repeated measures ANOVA, significant main effect of age F (1,11) = 8.372, $P = 0.015$; significant differences: middle-aged female control vs old female control, $P = 0.003$, post-hoc comparisons with Bonferroni corrections). The MDA concentrations were significantly higher in old female DIO mice than in their respective controls, with old female controls showing higher MDA concentrations than middle-aged controls (Fig. 4B; two-way repeated measures ANOVA, significant interaction diet × age F(1,11) = 5.573, $P = 0.038$, main effect of diet F (1,11) = 13.696, $P = 0.004$, main effect of age F(1,11) = 27.484, $P < 0.001$; significant differences: old female control vs old female DIO, $P = 0.034$; middle-aged female control vs old female control, $P < 0.001$, post-hoc comparisons with Bonferroni corrections). The superoxide anion concentrations were significantly higher in old female DIO mice in comparison with old female non-DIO controls, with old female controls showing higher superoxide anion amounts than middle-aged controls (Fig. 4C; two-way repeated measures ANOVA, significant main effect of diet F(1,11) = 38.509, $P < 0.001$, main effect of age F (1,11) = 14.439, $P = 0.003$; significant differences: old female control vs old female DIO, $P = 0.003$; middle-aged female control vs old female control, $P = 0.002$, post-hoc comparisons with Bonferroni corrections).

3.5. Life span

Male DIO mice showed a shorter life span than male non-DIO control mice (Fig. 5; Kaplan-Meier survival curve, $P = 0.043$). Male DIO mice also exhibited a shorter life span than female DIO mice (Fig. 5; Kaplan-Meier survival curve, $P = 0.033$). The differences were not statistically significant between female DIO mice and female non-DIO mice (Fig. 5).

4. Discussion

This is the first study, to the best of our knowledge, showing that the late adulthood diet-induced obesity onset led to significant impairments in behavior as well as in functions and redox state of peritoneal leukocytes of middle-aged male and female mice, with males being significantly more affected than females. Thus, middle-aged male DIO mice exhibited greater impairments in a variety of behavioral, immune and redox state parameters, and consequently showed a shorter life span than their female counterparts. In the middle-aged female DIO mice some parameters presented values similar to those in old female animals. These results confirm the state of accelerated immunosenescence as the consequence of the diet-induced obesity of the present study. In addition to obesity-related impairments of the immune system, other factors, such as insulin resistance (Frasca et al., 2017), dysbiosis and increased intestinal permeability (Boulangé et al., 2016), could also contribute to oxidative and inflammatory systemic stresses, and consequently to the process of accelerated aging in obese individuals.

We have previously shown that the high-fat diet intake is a good model to develop obesity in mice (Hunsche et al., 2016, 2018). This experimental animal model of obesity has been demonstrated to be capable of mimicking human obesity, regarding its etiologic aspects (Kanasaki and Koya, 2011). Thus, this is applied to both sexes, since in the present study, middle-aged male and female mice fed on a high-fat diet during 14 weeks of their late-adulthood displayed significantly higher body weight and fat mass than their respective non-DIO counterparts. Moreover, middle-aged male and female DIO mice showed higher levels of triglycerides and total cholesterol, which are well-known common features of obesity (Hunsche et al., 2016, 2018; Klop et al., 2013). Furthermore, the late adulthood diet-induced obesity onset differentially affected the total body fat of middle-aged male and female mice. Thus, female DIO mice showed significantly higher body fat mass in comparison with their male counterparts. In agreement, previous studies reported that females have a higher propensity to gain body weight and consequently to store more fat in the adipose tissue than males (Mauvais-Jarvis, 2015). In general, females, prior to menopause, tend to accumulate more fat in the subcutaneous adipose tissue, while males in the visceral adipose tissue. This visceral accumulation of fat has been associated with worsen metabolic outcomes in this sex (Palmer and Clegg, 2015). In agreement, in the present study, middle-aged male DIO mice showed higher plasma levels of glucose and total cholesterol than their female counterparts.

With respect to the results of behavioral tests, we observed lower muscular vigor, horizontal and vertical exploratory activities as well as higher anxiety in middle-aged male and female DIO mice than in their respective non-DIO controls. Middle-aged male and female DIO mice displayed a significantly lower central ambulation in the holeboard, which indicates a lower exploratory activity and higher anxiety behavior, since mice with anxiety tend to ambulate more in the peripheral area (close the walls) than in the central area (Simon et al., 1994). Moreover, middle-aged male and female DIO mice exhibited a longer time to complete the exploration of the three arms of the T-maze test than their respective non-DIO counterparts, which also indicates a lower exploratory activity. Regarding the elevated plus maze test, middle-aged male and female DIO mice displayed a lower percentage of time in the open arms. It is known that a lower activity in the open arms of the elevated plus maze apparatus indicate higher anxiety behavior (Walf and Frye, 2007). Moreover, the behavior of self-grooming and the presence of defecation, which could be considered as anxiety-related measures (Archer, 1973; Sake and Graybiel, 2003), were also significantly higher in middle-aged male and female DIO mice in comparison with their respective non-DIO controls. Previous studies indicate lower cognitive and exploratory activity as well as higher anxiety in DIO mice. However, in these studies, the high-fat diet was administered soon after weaning (André et al., 2014; Arnold et al., 2014; da Costa et al., 2015; Sharma and Fulton, 2013; Vallodolid-Acebes et al., 2011; Yamada et al., 2011). Studies in humans, including data from younger to older individuals, also observed an association between increased body weight and worse performance on gross and fine motor skills, equilibrium and muscular vigor (Deforche et al., 2009; Gentier et al., 2013; Kumar et al., 2008; Maffiuletti et al., 2007). Accordingly, it can be suggested that obese individuals perform worse gross motor tasks because a greater proportion of excess mass has to be supported or moved against gravity during these tasks. However, the worse performance on fine motor tasks cannot be solely explained by excess fat mass, given that fine motor skills are not directly influenced by the amount of mass (Gentier et al., 2013). In this sense, previous studies suggest that obese individuals show lower neural efficiency and slowed integration and processing of information (Gunning-Dixon and Raz, 2000; Mehta and Shortz, 2014). In addition, an association has been found between increased muscular fat infiltration in obese and aged individuals and lower muscle quality and strength (Marcus et al., 2010; Moore et al., 2014). The results of the present study also showed sex differences in the evaluation of behavioral tests, with males being more affected than females. Thus, middle-aged male DIO mice showed a worse performance than their female counterparts in the tests that evaluated motor coordination, equilibrium, muscular vigor, vertical and horizontal exploratory activities and anxiety-like behavior. However, most of the previous studies on obesity performed in rodents have used a single sex (André et al., 2014; da Costa et al., 2015; Sharma and Fulton, 2013; Vallodolid-Acebes et al., 2011; Yamada et al., 2011), thus there is little information comparing the behavioral effects of obesity in both sexes. Nevertheless, in agreement with our data, a previous study considering sex differences in the performance of behavioral tests, showed males more vulnerable than females to high-fat diet (HFD)-induced impairments, such as learning functions and synaptic plasticity

(Hwang et al., 2010). The mechanisms underlying this higher impact of HFD and obesity in males than in females remain unknown, but some evidence indicates that estrogen could have a positive effect in protecting female mice. In this sense, male mice treated with estradiol showed less adiposity than controls (Salinero et al., 2018).

With respect to the functions studied in the peritoneal macrophages, middle-aged male and female DIO mice showed diminish chemotactic and phagocytic capacities of these cells in comparison with their respective non-DIO counterparts. These results are in agreement with those obtained in our previous study in which obesity in early adulthood was induced during the adolescent period (Hunsche et al., 2016). Thus, both the different ages of obesity onset (early and late adulthoods), resulted in impaired innate immune response. In addition, differently from our previous study, the present study also evaluated the immune effects of obesity in males. The results showed that middle-aged male DIO mice had lower chemotactic and phagocytic indexes than their respective female counterparts. Moreover, sex differences were also observed in non-DIO controls, with males showing lower values than females in some functions. Previous evidence confirms that males display a lower innate immune response against infection in comparison with females (Jaillon et al., 2017). Thus, it is possible that obesity could further exacerbate these impairments found in the innate immunity of males.

Regarding the anti-tumor activity natural killer (NK) of peritoneal leukocytes, middle-aged male and female DIO mice showed a lower response than their non-DIO counterparts, although no sex differences were observed in this function. The migration capacity of lymphocytes to a chemoattractant was significantly lower in middle-aged male and female DIO mice in comparison with their respective non-DIO controls. Similarly, the proliferation of lymphocytes in ConA and LPS-stimulated conditions, two mitogens for T and B lymphocytes, respectively, were also lower in middle-aged male and female DIO mice than in their non-DIO counterparts. These results are in agreement with those obtained in adult DIO female mice that ingested the diet rich in fat during adolescence (Hunsche et al., 2016). Thus, both early and late adulthood obesity onsets resulted in impaired functions of lymphocytes. Although middle-aged male control mice had lower values than middle-aged female control mice in chemotaxis of lymphocytes, male DIO mice presented higher chemotactic index and LPS-stimulated proliferation of lymphocytes than female DIO mice. The higher response of these immune functions in males could be possibly interpreted as a compensatory mechanism to counteract the lower response found in innate immune cell functions (Franceschi et al., 1995), especially in the context of an obesity state.

Oxidative stress, which is generated by an imbalance between oxidants and antioxidant in favor of oxidants, is associated with the development of obesity, aging and health complications (Bauer and De la Fuente, 2016; Marseglia et al., 2014). The current results demonstrated that middle-aged male and female DIO mice showed higher values of oxidants, such as xanthine oxidase activity, lipid peroxidation (MDA) concentrations, and GSSH/GSH ratios as well as lower values of antioxidant defenses (such as catalase activity and GSH concentrations). These results are partly in concordance with our previous experiment in which it was found increased oxidative stress in adult DIO female mice (Hunsche et al., 2016). However, differently from these previous data, the activity of glutathione peroxidase (an antioxidant enzyme) was significantly higher in middle-aged DIO animals in comparison with their respective non-DIO counterparts. In this sense, it is possible that the higher activity of this antioxidant enzyme could be explained by a compensatory mechanism in an attempt to restore the appropriate redox state of DIO animals. In fact, the activity of this enzyme can increase or decrease in oxidative stress situations depending on the moment of their evolution and the amount of peroxides generated (Liu et al., 2004; Yan and Harding, 1997). Sex differences were observed in the redox parameters studied of middle-aged non-DIO animals, with males being more affected than females. Thus, in response to the late age of obesity onset, middle-aged male DIO mice showed higher values of xanthine oxidase and lipid peroxidation (MDA) and lower values of glutathione peroxidase activities in comparison with their female counterparts. Previous studies indicate that males are more susceptible to present higher oxidative stress than females (Baeza et al., 2011); however this condition is still controversial and contradictory data have been reported (Kander et al., 2017). Moreover, there is a lack of evidence about sex differences in the redox state of obese individuals.

At old age, female DIO mice continuous to exhibit some impaired immune functions and redox state parameters than old female non-DIO controls. However, differently from our previous data (in the early adulthood obesity onset) (Hunsche et al., 2016), the late adulthood diet-induced obesity onset resulted in no significant differences in some immune functions (such as anti-tumor NK cell activity and LPS-stimulated proliferation of lymphocytes), redox state (xanthine oxidase activity, GSSG/GSH ratio, catalase and glutathione peroxidase activities) and in the life span between female DIO mice and female non-DIO mice. Thus, the early adulthood obesity onset, in which adolescent mice were exposed to a high-fat diet, had more long-lasting deleterious effects in the immune system and consequently in the life span than a later obesity onset. Increasing evidence suggest that adolescence is a critical period in which the nervous and immune systems are still experiencing developmental changes and thus are more susceptible to stresses, such as an increased consumption of high-fat diet (Boitard et al., 2012; Holder and Blaustein, 2014; Simon et al., 2015; Spear, 2000).

Although several innate and adaptive immune functions were analyzed here, other relevant aspects of immunosenescence should be considered in future studies. Thus, acquisition of the senescence-associated phenotype (SASP), immunophenotyping aging subsets, and intracellular signaling pathways, could confirm the accelerated immunosenescence state of obese individuals.

In conclusion, the results of the current study provide evidence that the late adulthood diet-induced obesity onset leads to impairments in the functions of the nervous (behavior) and immune system (function and redox state of peritoneal leukocytes) of middle-aged male and female mice. Sex differences were found in some of the behavioral, immune function and redox state parameters, being males significantly more affected than females. Since many of these functions have been proposed as markers of health, rate of aging and predictor of longevity (De la Fuente and Miquel, 2009, Martínez de Toda et al., 2016), the results show a worst health status in middle-aged female and especially male DIO mice and, consequently, a lower life span. In addition, the late adulthood obesity partially exacerbated the age-related impairments in immune cell functions and redox state of old female mice.

Acknowledgments

This work was supported by grants from the Research Group of Madrid Complutense University (910379ENEROINN) and FIS (PI15/01787) of Institute de Salud Carlos III – Fondo Europeo de Desarrollo Regional (ISCIII-FEDER). C.H. is the recipient of a PhD fellowship from CNP-q-Brazil.

Conflict of interest

The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbi.2019.01.010.

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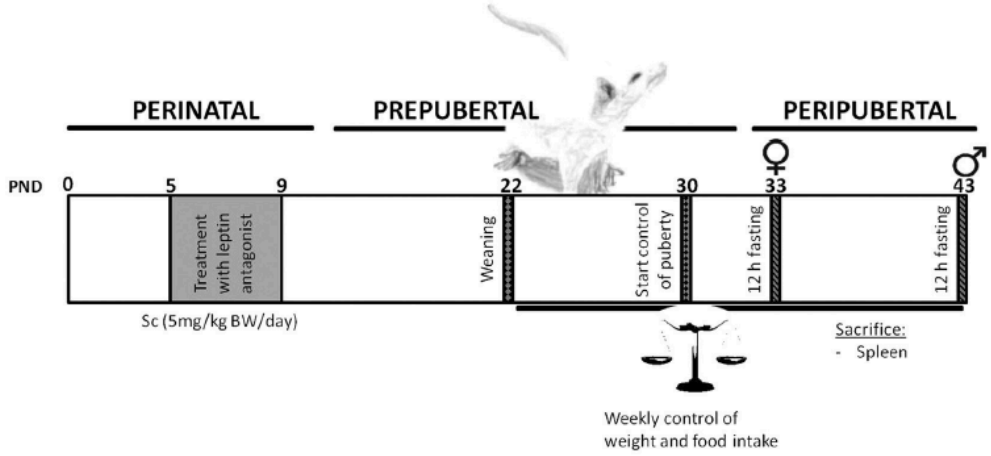
3.2. EFFECTS OF THE BLOCKAGE OF THE NEONATAL LEPTIN SURGE (PND5-9) ON THE IMMUNE FUNCTION AND REDOX/INFLAMMATORY STATE OF MALE AND FEMALE RATS AT DIFFERENT AGES

3.2.1. Effects of the blockage of the neonatal leptin surge (PND5-9) on the redox/inflammatory state in the spleen, hypothalamus and white adipose tissue of peripubertal/adolescent male and female rats.



Experimental design

From PND5 until PND9 rats were injected subcutaneously with 5 mg/kg bodyweight (bw) of rat pegylated super leptin antagonist (mutant D23L/L39A/D40A/F41A). In order to avoid the stress effect caused by marking the animals at this early age, each litter was treated with either vehicle (controls) or antagonist. The animals received one injection per day at 9:00 am. Control rats were injected with the same volume of distilled water as the vehicle (2.5 ml/kg). After each injection the animals were immediately returned to their mothers. Each experimental group consisted of 12 animals. To avoid/minimize a possible litter effect, all experimental groups contained animals from at least three different litters. All rats were left undisturbed until weaning at PND22 at which time they were separated into 4 rats of the same sex per cage. Body weight was

monitored weekly until 5 days before sacrifice. Each experimental group consisted of 12 rats. Female (PND33) and male (PND43) rats were sacrificed after a 12h fast by decapitation. The hypothalamus, white adipose tissue (WAT) and spleen were rapidly and aseptically removed. The hypothalamus and WAT were frozen and stored at -80ºC until the assays were performed. The spleen was freed of fat, weighed, and divided into two parts. One of them was stored at -80ºC until the assays were performed. The other was minced with scissors, and gently pressed through a mesh screen to obtain the cell suspension. The levels of pro-inflammatory (TNF-α) and anti-inflammatory (IL-10, IL-13) cytokines released into the supernatants of spleen leukocyte cultures, after 48 hours of incubation with ConA or LPS, were collected and stored at -80°C until used for cytokine analysis.

Main results

Body and spleen weight measurements

There were no significant differences in body weight between control and treated animals during the treatment period and/or at the end of the experiment. Regarding spleen weights, a significant effect of the neonatal leptin antagonist treatment was found in both sexes, with treated males exhibiting lower spleen weights and treated females showing higher spleen weights when comparing with their respective control counterparts.

Oxidative stress parameters

Both males and females treated neonatally with the leptin antagonist showed significantly lower values of three enzymes analysed: superoxide dismutase, catalase, and glutathione peroxidase activities. In addition, male and female treated animals showed higher glutathione reductase activity. No significant differences were found in the GSSG/GSH ratios.

Cytokine levels

The concentrations of the anti-inflammatory cytokine IL-10 released by spleen leukocytes from treated animals were significantly lower in the presence of LPS and also in the presence of

ConA. The treatment with the leptin antagonist resulted in lower values of the anti-inflammatory cytokine IL-13 in male leukocytes stimulated with LPS and in female leukocytes stimulated with ConA. The concentrations of the pro-inflammatory cytokine TNF-α were also modified by the neonatal treatment with the leptin antagonist. In the presence of LPS, leukocytes of treated males released lower concentrations, and those of females higher concentrations of this cytokine than their corresponding control counterparts. In the presence of ConA, treated males showed higher TNF-α values, whereas no differences were found in females.

Hypothalamic mRNA levels

The leptin antagonist had no significant effect on catalase, superoxide dismutase and glutathione reductase mRNA levels, although there was a tendency to increase mRNA levels of glutathione peroxidase in males. Regarding cytokine mRNA levels, there was a trend to increase TNF-α values in males and a significant decrease in females due to the treatment. No effect was found in IL-10 mRNA levels.

Adipose tissue mRNA levels

The levels of catalase and glutathione reductase mRNA were decreased by the leptin antagonist in males, with no significant effect in females. A similar effect was found in superoxide dismutase mRNA values where the treatment trended towards a decrease in males. No effects were found in glutathione peroxidase mRNA levels. In addition, increases in IL-1β and IL-6 were found in treated males with no significant effect in female groups.

Partial conclusions

The results of this experiment indicate that the blockage of the neonatal leptin surge results in an altered redox/inflammatory state of the spleen, hypothalamus and white adipose tissue in peripubertal/adolescent male and female rats.

Molecular and Cellular Endocrinology 454 (2017) 125–134

Contents lists available at ScienceDirect

Molecular and Cellular Endocrinology

journal homepage: www.elsevier.com/locate/mce

Administration of a leptin antagonist during the neonatal leptin surge induces alterations in the redox and inflammatory state in peripubertal /adolescent rats

Virginia Mela [a, *], Oskarina Hernandez [a], Caroline Hunsche [a], Francisca Diaz [b, c], Julie A. Chowen [b, c], Mónica De la Fuente [a]

[a] Department of Physiology (Animal Physiology II), Faculty of Biology, Complutense University, Madrid, Spain
[b] Department of Endocrinology, Hospital Infantil Universitario Niño Jesús, Department of Pediatrics, Universidad Autónoma de Madrid, Instituto de Investigación Sanitaria Princesa (IP), Madrid, Spain
[c] CIBER Fisiopatología de Obesidad y Nutrición, Instituto Carlos III, Madrid, Spain

ARTICLE INFO

Article history:
Received 19 January 2017
Received in revised form
28 April 2017
Accepted 17 June 2017
Available online 19 June 2017

Keywords:
Neonatal leptin
Leptin antagonist
Antioxidant defenses
Proinflammatory and anti-inflammatory cytokines
Peripubertal rats

ABSTRACT

The importance of the neonatal leptin surge in rodents in neurodevelopmental processes has aroused curiosity in its implication in other physiological systems. Given the role of leptin in neuro-immune interactions, we hypothesized that the neonatal leptin surge could have an effect on the oxidative and inflammatory stress situations of both systems. We blocked the neonatal leptin surge by a leptin antagonist and measured several parameters of oxidative and inflammatory stress in the spleen, hypothalamus and adipose tissue of peripubertal/adolescent rats. The treated rats showed lower activity of several antioxidant enzymes in the spleen and their leukocytes released lower levels of mitogen-stimulated IL-10 and IL-13 and higher levels of TNF-alpha. In conclusion, the neonatal leptin surge may have a key role in the establishment of adequate redox and inflammatory states in the immune system, which is important for the generation of adequate immune responses and to obtain and maintain good health.

1. Introduction

Leptin, an adipokine produced mainly by adipocytes, is a pleiotropic hormone involved in many physiological processes such as food intake and energy balance, immune system functions, reproduction, reward, stress and neurodevelopment (Bellefontaine et al., 2014; Folch et al., 2012; Meyer et al., 2014). In rodents, there is a postnatal leptin surge, that in male mice begins around postnatal day (PND) 5 and peaks between PND9 and 10 (Ahima and Hileman, 2000). In rats, leptin increases between PND4 and 7, is elevated between PND7 and 10 and then declines by PND14 (Delahaye et al., 2008). This leptin surge has been implicated in hypothalamic development by modifying neuronal outgrowth and synaptic connectivity, as well as neurogenesis and neuronal and glial survival (Bouret, 2013). The timing and magnitude of this surge appears to be important for normal metabolic control and weight gain in the adult animal (Ahima et al., 1998; Attig et al., 2008; Cottrell et al., 2009; Delahaye et al., 2008; Yura et al., 2005). Most studies on the neurotrophic role of leptin during its neonatal peak have focused on the hypothalamus (Bouret, 2013; Bouret et al., 2004; Garza et al., 2008; V Mela et al., 2012a,b; Pinto et al., 2004; Udagawa et al., 2006a, 2006b; Viveros et al., 2010). In fact, this brain structure has traditionally been the focus of studies related to central leptin actions. For instance, we recently found that administration of a pegylated super leptin antagonist from PND 5 to 9, coincident with the neonatal leptin surge, affects the gene expression of growth factors, glial proteins, and neuropeptides involved in the control of metabolism and reproduction in the hypothalamus of peri-pubertal male and female rats (Mela et al., 2015).

Currently, there are abundant studies that confirm the bidirectional communication between the homeostatic systems, namely the nervous, endocrine and immune systems, which is mediated by neurotransmitters, hormones and cytokines through the presence of their receptors on the cells of all three systems. Thus, any influence exerted on one of these systems will have an effect on the

* Corresponding author. Department of Physiology (Animal Physiology II), Faculty of Biology, Complutense University, 28040 Madrid, Spain.
E-mail address: virginiamelarivas@gmail.com (V. Mela).

http://dx.doi.org/10.1016/j.mce.2017.06.018
0303-7207/

others and *vice versa* (Vida et al., 2014; Wrona, 2006). Leptin receptors are highly expressed in immune cells such as monocytes, macrophages, neutrophils and lymphocytes (Goldenberg, 2014) and, thus, leptin affects almost all cells of the immune system (Zabeau et al., 2014), modulating immune cell functions and cytokine networks, which may contribute to the protection from infections (Conde et al., 2014; Procaccini et al., 2013). Consequently, a decrease in leptin release or a disruption of its actions could be deleterious for the immune response. Indeed, Farooqi et al (Sadaf Farooqi et al., 2002)., showed that individuals with leptin deficiency were more likely to suffer infections due to the impairment in T cell proliferation and cytokine release. In leptin deficient ob/ob mice, dendritic cells are less effective in the stimulation of allogenic T cells (Macia et al., 2006) and the balance of Th1/Th2 cytokines is deregulated, changing the sensitivity to autoimmune diseases (Zabeau et al., 2014).

Leptin can also promote the release of reactive oxygen species (ROS) and ROS have an important role as signaling molecules in the central nervous system, and more particularly in the hypothalamus. In POMC neurons, ROS levels that may be regulated among other factors by leptin, appear to play a crucial role in modulating cellular responses involved in the regulation of energy metabolism. In fact, the short-term hypothalamic ROS peak generated by metabolic signals (nutrients and hormones) appears to be fundamental to elicit a proper behavioral, endocrine and autonomic response to nutrient intake (Diano, 2013; Drougard et al., 2015). In an experimental model of *L. donovani* infection, leptin induced macrophage phagocytic activity by enhancing intracellular ROS generation, which helps in phagolysosome formation and oxidative killing of the parasite (Dayakar et al., 2016). ROS production is important for appropriate body function, including the immune response. However, when there is an excessive increase in ROS levels, due to an enhancement in their production or a decrease in the antioxidant defenses that neutralize the excess of ROS, a situation of oxidative stress appears, and these ROS can be deleterious for the organism causing oxidation of macromolecules (Gyengesi et al., 2012; Vida et al., 2014). It is known that oxidation and inflammation are related processes and that an excess of oxidant and inflammatory compounds maintained through time produces a situation of oxidative and inflammatory stress, which deregulates immune function and is the cause of aging, illness and death (Vida et al., 2014). Alterations in immune and inflammatory responses are present in leptin- and leptin-receptor deficient animals, as well as during starvation and malnutrition, two conditions characterized by low levels of circulating leptin (Fantuzzi and Faggioni, 2000).

In spite of the findings described above, the specific role of the neonatal leptin surge on the development and establishment of a correct redox state and a regulated inflammatory response has not been investigated. To address this question, diverse parameters related to oxidative and inflammatory stress were analyzed in the spleen, as well as in the hypothalamus and white adipose tissue of peripubertal/adolescent male and female rats that received daily injections of a rat pegylated super leptin antagonist from PND5 until PND9, the time period of the neonatal leptin surge. This allows a first approach to determine if this leptin surge has a role in the redox and inflammatory states of peripubertal/adolescent animals, at least at the level of the immune and nervous systems as well as in white adipose tissue.

2. Materials and methods

2.1. Animals

Adult Wistar rats were purchased from Harlan Interfauna Ibérica S.A. (Barcelona, Spain) and allowed to acclimatize for 2 weeks before mating. One male was placed in a cage with two females for 10 days. On the day of birth (PND0), litters were culled to eight pups per dam (four males and four females). No cross-fostering was employed. Thus, only litters with at least 4 pups of each sex at birth were used. Rats were maintained at a constant temperature (22 ± 1 °C) and humidity (50± 2%) in a reversed 12-h light-dark cycle (red light on at 08:00 and white light on at 20:00). Pregnant rats were given free access to rat chow (commercial diet for rodents; A03, Safe, Augy, France) and water. The animals used in this study were the same as those used in a recently published paper that focused on the effects of the same leptin antagonist treatment on hypothalamic systems related to reproduction (Mela et al., 2015). As our aim in that study was to investigate the pubertal transition, right before the appearance of external phenotypic signs of puberty, and because the timing of puberty differs between the sexes, females were euthanized on PND33 and males on PND43. These studies were approved by the local ethics committee and complied with the Royal Decree 1201/2005 (BOEn° 252) pertaining to the protection of experimental animals and with the European Communities Council Directive (86/609/EEC). The experiments were conducted in accordance with the guidelines and protocols of the Royal Decree 53/2013 regarding the care and use of laboratory animals for experimental procedures, and were approved by the Committee for Animal Experimentation of the Complutense University of Madrid.

2.2. Leptin antagonist treatment

From PND5 until PND9 rats were injected sc with 5 mg/kg bodyweight (bw) of rat pegylated super leptin antagonist (mutant D23L/L39A/D40A/F41A), a gift of Protein Laboratories (Rehovot, Israel), which was prepared as described in Jamroz-Wiśniewska et al. (2014). In order to avoid the stress effect caused by marking the animals at this early age, we treated each litter with either vehicle (controls) or antagonist. The animals received one injection per day at 9:00. Control rats were injected with the same volume of distilled water as vehicle (2.5 ml/kg). After each injection the animals were immediately returned to their mothers. Each experimental group consisted of 12 animals. To avoid/minimize a possible litter effect all experimental groups contained animals from at least three different litters.

2.3. Body weight increase

All rats were left undisturbed until weaning at PND22 at which time they were separated into 4 rats of the same sex/cage. Body weight was monitored weekly until 5 days before sacrifice. Each experimental group consisted of 12 rats.

2.4. Tissue collection

Female (PND33) and male (PND43) rats were sacrificed after a 12 h fast by decapitation. The hypothalamus, white adipose tissue (WAT) and spleen were rapidly and aseptically removed. The hypothalamus and WAT were frozen and stored at −80 °C until the assays were performed. The spleen was freed of fat and divided into two parts. One of them was stored at −80 °C until the assays were performed. The other was minced with scissors, and gently pressed through a mesh screen (Sigma, St. Louis, and USA) to obtain the cell suspension.

2.5. Antioxidant enzymes

2.5.1. Superoxide dismutase (SOD) activity

Total SOD activity was measured (n = 8–11) by a modified

method described by Marklund and Marklund (1974), with slight modifications (Alvarado et al., 2006). The tissue samples (50 mg/ml) in Tris–HCl buffer (50 mM, pH 8.2) were homogenized and centrifuged at 3200 g for 20 min at 4 °C. Supernatant (750 μl) was mixed with an ice-cold ethanol-chloroform mixture (5:3 v/v; 600 μl) and immediately shaken for 60 s and centrifuged at 2500 g for 10 min at 4 °C. Supernatant (SOD extract; 25 μl) was mixed with Tris–HCl buffer (50 mM, pH 8.2) containing DTPA (1 mM) and pyrogallol (24 mM prepared in HCl 10 mM; 25 μl). The enzymatic activity was measured spectrophotometrically at 420 nm through the oxidation of pyrogallol. One unit of activity is defined as the amount of enzyme causing a 50% inhibition of the autoxidation of pyrogallol at 25 °C. The protein contents of the same samples were evaluated following bicinchoninic acid protein assay kit protocol (Sigma-Aldrich, Spain). The results were expressed as arbitrary units (U) of enzymatic activity per milligram of protein (AU SOD/mg protein).

2.5.2. Catalase (CAT) activity

Catalase activity was determined (n = 6–11) following the method described by Aebi (1984) with modifications previously introduced by Alvarado et al. (2006), and based on the decrease of the absorbance at 240 nm because of the decomposition of H_2O_2 by the enzyme. The assays were performed using aliquots of the homogenized tissue samples (50 mg/ml in tampon phosphate 66 mM, pH 7.5) and centrifuged at 3200 g for 20 min at 4 °C. The supernatant extract (30 μl) was mixed with 670 μl of H_2O_2 (14 mM in phosphate buffer 66 mM). The enzymatic activity was measured spectrophotometrically for 80 s at 240 nm through the decomposition of H_2O_2 into $H_2O + O_2$. One unit of CAT was defined as the amount of enzyme that liberates half of the peroxide oxygen from H_2O_2 solution in 80 s at 25 °C. Protein content was evaluated following the same bicinchoninic acid protein assay kit protocol. The results were expressed as units (U) of enzymatic activity per milligram of proteins (U CAT/mg protein).

2.5.3. Glutathione peroxidase (GPx) activity

The glutathione peroxidase (GPx) activity was determined (n = 7–11) according to the technique described by Lawrence and Burk (1976). The assays were performed with aliquots of the homogenate tissue samples (50 mg/ml in tampon phosphate 50 mM, pH 7.5) and centrifuged at 3200 g for 20 min at 4 °C. The total activity was determined using cumene hydroperoxide (Sigma-Aldrich), which carried out the oxidation of the glutathione regenerated by the addition of B-nicotinamide adenine di-nucleotide phosphate, in its reduced form (β-NADPH, Sigma-Aldrich, Spain), in the presence of glutathione reductase (Sigma-Aldrich). The reaction was followed spectrophotometrically by the decrease of the absorbance at 340 nm. The protein contents were evaluated following the previously mentioned protocol. The results are expressed as milliunits of enzymatic activity per milligram of proteins (mUGPx/mg protein).

2.5.4. Glutathione reductase (GR) activity

The glutathione reductase (GR) activity was measured (n = 8–10) by the method described by Massey and Williams (1965). The assays were performed with aliquots of the homogenate tissue samples (50 mg/ml in tampon phosphate 50 mM, pH 7.5 with 6.3 mM EDTA) and centrifuged at 3200 g for 20 min at 4 °C. The total activity was measured through the oxidation of NADPH spectrophotometrically at 340 nm. The protein contents of the same samples were again evaluated following the previously described protocol. The results are expressed as milliunits of enzymatic activity per milligram of proteins (mUGR/mg protein).

2.5.5. Total glutathione, GSH and GSSG concentrations

The total glutathione (GSH plus GSSG) was measured (n = 10–12) by fluorometry, according to the Hissin and Hilf method (Hissin and Hilf, 1976), with some modifications (Schultz et al., 2010), in which GSH was sequentially oxidized by o-phthaldialdehyde and reduced by NADPH in the presence of GR. The oxidized glutathione level (GSSG) was determined by masking GSH with N-ethylmaleimide. The protein contents of the same samples were evaluated following the previously mentioned protocol. The amounts of GSH and GSSG were calculated using standard curves, which were prepared using a glutathione solution (glutathione reductase and oxidized form 1 μg/ml) (Sigma-Aldrich, Spain), and expressed in nmol per milligram of protein (nmol/mg protein). The redox index was calculated by dividing the concentration of GSSG by that of GSH.

2.6. Cytokine levels released by spleen leukocytes

Spleen cell suspensions were centrifuged in a gradient of Ficoll-Hypaque (Sigma-Aldrich, Spain) with a density of 1.070 g/ml. The cells were recollected from the interface and resuspended in RPMI 1640 medium enriched with L-glutamine (PAA, Pasching Austria) and supplemented with 10% heat-inactivated fetal calf serum (Gibco, Canada) and gentamicin (10 mg/ml, Gibco) (complete medium). Aliquots of 200 μl of mononuclear cell suspension adjusted to 1×10^5 cells/ml complete medium were cultured in 96-well plates in the presence of lipopolysaccharide (LPS) (*Escherichia coli* 055:B5, 1 μg/ml per well; Sigma-Aldrich, Spain) or concanavaline A (ConA, 1 μg/mL per well; Sigma Sigma-Aldrich, Spain). After 48 h of incubation at 37 °C in a sterile and humidified atmosphere of 5% CO2, 100 μl of culture supernatant from each well was collected and stored at −80 C until used for cytokine analysis. Levels of proinflammatory (TNF-α) and anti-inflammatory (IL-10, IL-13) cytokines were measured (n = 6–8) simultaneously by Luminex xPONENT (Rat Bio Plex Pro Cytokine assays using magnetic beads, U.S, BIO-RAD). The results were expressed as pg/mL.

2.7. Quantitative real-time PCR

To determine whether leptin antagonist treatment affected the redox state in other tissues, apart from spleen, we measured the mRNA expression profile of redox enzymes on hypothalamus and subcutaneous adipose tissue by quantitative real-time PCR.

Total mRNA from hypothalamus and subcutaneous adipose tissue was isolated with Trizol® Reagent (Invitrogen Carlsbad, CA, USA) following the manufacturer's recommendation. A high-capacity cDNA RT kit (Applied Biosystems, Foster City, CA) was used to transcribe 1 μg of total mRNA on a thermal cycler Tetrad 2 (Peltier Thermal Cycler, Bio-Rad). cDNA template amplification was performed by using a TaqMan Universal PCR Master Mix (Applied biosystems) on a thermal cycler ABI PRISM 7900HT Sequence Detection System (Applied Biosystems) and mRNA levels of catalase (CAT, Rn00560930_m1), superoxide dismutase 2 (SOD, Rn00690588_g1), glutation peroxidase (GPx, Rn00577994_g1) and glutation reductase (Grs, Rn01482159_m1) were assessed by TaqMan Gene Expression Assay-on-demand kits (Applied Biosystems) for each gene in both tissues. Each sample was run in duplicate (n = 5–6). Various housekeeping genes were analyzed and the gene that showed no variation between groups was chosen to normalize the data in that tissue. The results were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Rn99999916_s1) in the hypothalamus, and Rps18 (Rn01428915_g1) and PPIA (Rn00690933) for adipose tissue. The ΔΔCT was used to determine relative mRNA levels. Statistics were performed using ΔΔCT values.

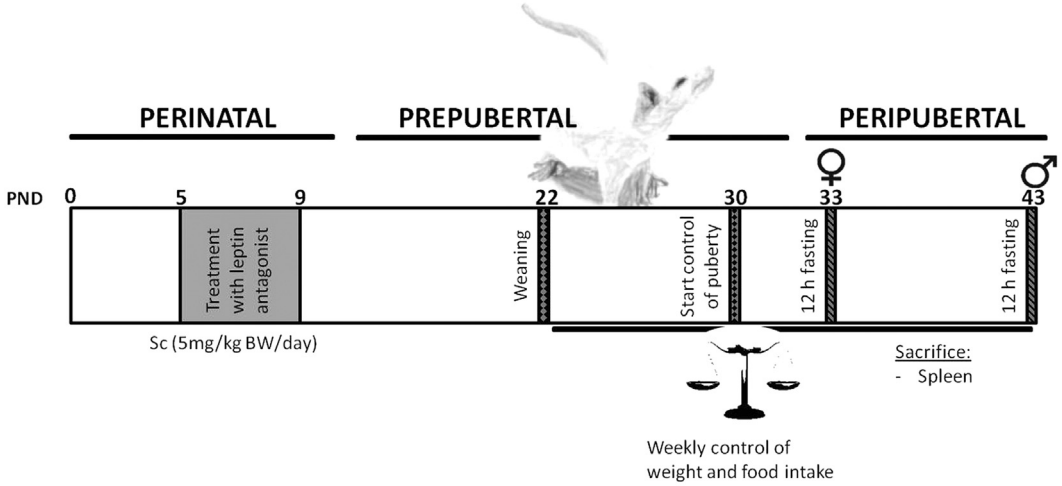


Fig. 1. Experimental design diagram.

2.8. Statistical analysis

Data were analyzed by SPSS for Windows, release 15.0. Normality was checked by Shapiro-Wilks's test ($p > 0.05$). Data were analyzed by one-way analysis of variance (ANOVA) and for the change in body weight during the leptin antagonist treatment an ANOVA with repeated measures was performed. As males and females were of a different chronological age due to the differential timing of puberty onset, data were not directly compared between sexes (see Fig. 1).

3. Results

3.1. Body and spleen weights

There were no significant differences between control and treated animals during the treatment period and/or at the end of the experiment (Fig. 2). Regarding spleen weights, a significant effect of the neonatal leptin antagonist treatment was found in both sexes [Males: $F(1,22) = 22.012$; $p < 0.001$. Females: $F(1,17) = 22.380$; $p < 0.001$] with treated males exhibiting lower spleen weights and treated females showing higher spleen weights when comparing with their respective control counterparts (Fig. 2).

3.2. Parameters of oxidative stress

Both, males and females treated neonatally with the leptin antagonist showed significantly lower activity of the three enzymes analyzed, CAT [Males: $F(1,20) = 3.027$; $p < 0.001$. Females: $F(1,15) = 19.732$; $p < 0.001$] (Fig. 3A), SOD [Males: $F(1,19) = 8.625$; $p < 0.01$. Females: $F(1,17) = 4.752$; $p < 0.05$] (Fig. 3B) and GPx (Fig. 4A), [Males: $F(1,19) = 42.964$; $p < 0.001$. Females: $F(1,16) = 11.034$; $p < 0.01$]. In addition, male and female treated animals showed higher GR activity (Fig. 4B) [Males: $F(1,18) = 73.738$; $p < 0.001$. Females: $F(1,16) = 29.017$; $p < 0.001$]. No significant differences were found in the GSSG/GSH ratios (Fig. 5).

3.3. Cytokine levels

The levels of the anti-inflammatory cytokine IL-10 released by spleen leukocytes from treated animals were significantly lower in the presence of LPS [Males: $F(1,10) = 682.412$; $p < 0.001$. Females: $F(1,14) = 17.044$; $p < 0.01$] and also in the presence of ConA [Males: $F(1,14) = 44.522$; $p < 0.001$. Females: $F(1,14) = 8.256$; $p < 0.05$] (Fig. 6). The treatment with the leptin antagonist resulted in lower levels of the anti-inflammatory cytokine IL-13 in male leukocytes stimulated with LPS (Fig. 7A) [$F(1,14) = 28.767$; $p < 0.001$] and in female leukocytes stimulated with ConA (Fig. 7B) [$F(1,13) = 13.506$; $p < 0.01$].

As shown in Fig. 8, the levels of the proinflammatory cytokine TNF-α were also modified by the neonatal treatment with the leptin antagonist. In the presence of LPS, leukocytes of treated males released lower levels, and those of females higher levels of this cytokine than their corresponding control counterparts [Males: $F(1,12) = 14.960$; $p < 0.01$. Females: $F(1,14) = 22.464$; $p < 0.001$]. In the presence of ConA, treated males showed higher TNF-α levels [Males: $F(1,25) = 6.639$; $p < 0.05$], whereas no differences were found in females (Fig. 8).

3.4. Hypothalamic mRNA levels

The leptin antagonist had no significant effect on catalase, SOD and GR mRNA levels although, as Table 1 shows, there was a tendency to increase mRNA levels of GPx in males [$F(1,10) = 4.57$; $p = 0.058$].

Regarding cytokine mRNA levels, there was a trend to increase TNFa levels in males and a significant decrease in females due to the treatment [Males: $F(1,8) = 3.70$; $p = 0.09$, Females: $F(1,8) = 6.73$; $p < 0.05$]. No effect was found on IL10 mRNA levels.

3.5. WAT mRNA levels

The levels of catalase and GR mRNA were decreased by the leptin antagonist in males [catalase: $F(1,8) = 7.46$; $p < 0.05$, GR: $F(1,9) = 7.12$; $p < 0.05$] with no significant effect in females. A similar effect was found in SOD mRNA levels where the treatment trended towards a decrease in the levels of this enzyme in males [$F(1,9) = 3.63$; $p = 0.06$]. No effects were found in GPx mRNA levels (Table 2).

As Table 2 shows, an increase in IL1b [$F(1,10) = 12.19$; $p < 0.01$] and IL6 [$F(1,7) = 24.66$; $p < 0.005$] was found in treated males with no significant effect in female groups.

4. Discussion

The observation that the activity of diverse antioxidant enzymes

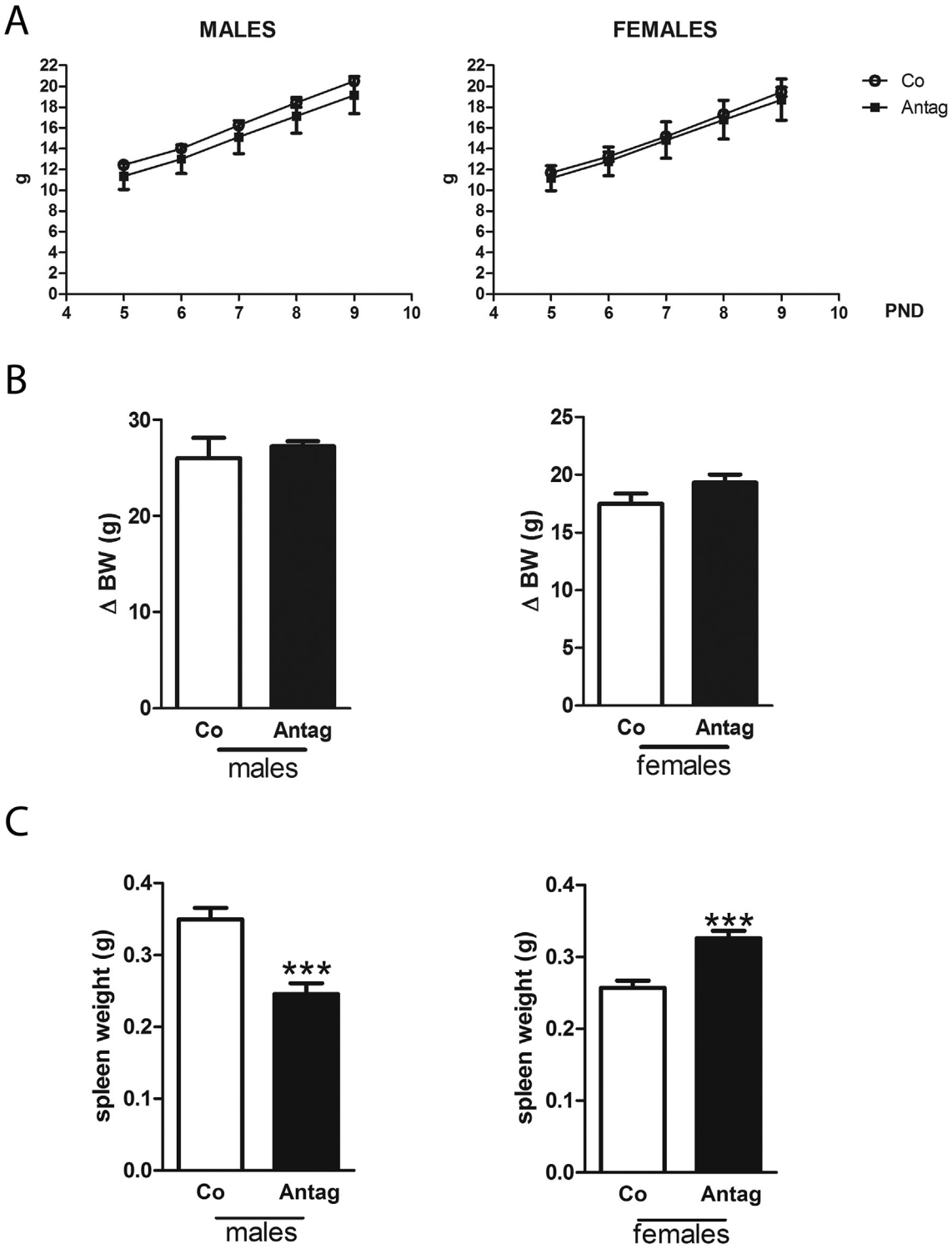


Fig. 2. Changes in body weight gain during the treatment (A) and at the end of the experiment (B) and spleen weight(C) at the time of sacrifice in peripubertal male and female rats treated neonatally with a leptin antagonist (Antag) or vehicle (Co). Data expressed as mean ± S.E.M. (n = 8–12 per experimental group). One way ANOVA: ***p < 0.001.

and the levels of pro-inflammatory and anti-inflammatory cytokines are modified during the peripubertal/adolescent phase in the neonatally treated rats provides the first evidence for a programming role of the physiological leptin surge in the redox and inflammatory state of the animal later in life.

Similar to that described in mice (Ahima et al., 1998), rats also experience a plasma surge of leptin during the neonatal period (Delahaye et al., 2008). In these animals, leptin increases between PND4 and 7, is elevated between PND7 and 10, declining by PND14. Therefore, the present leptin antagonist treatment was administered precisely during the time of the leptin surge. Leptin appears to play a key role in immune and inflammatory responses (Conde

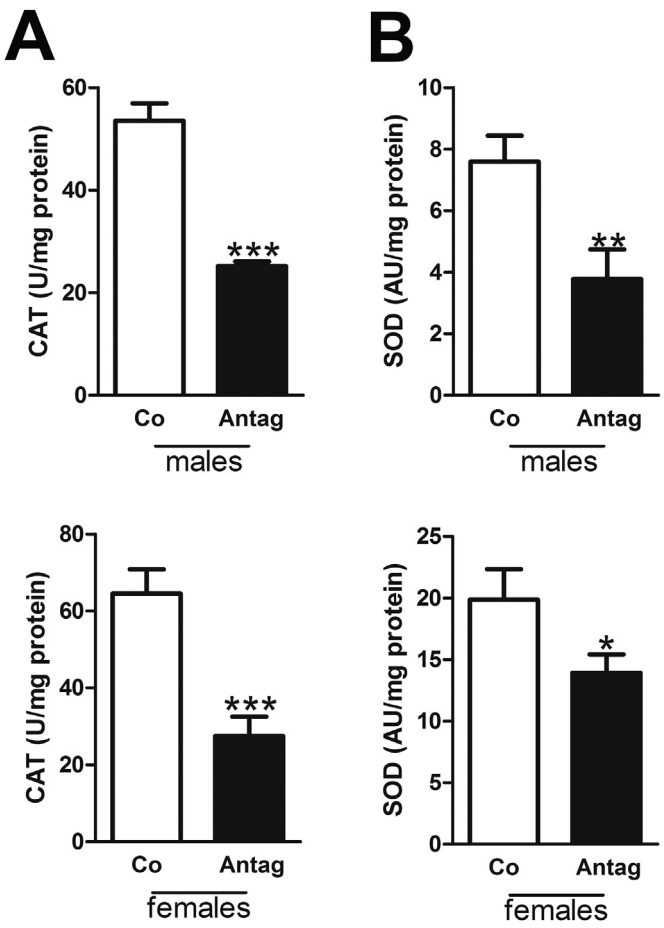


Fig. 3. Changes in CAT (A) and SOD (B) activity in spleen homogenate (data expressed as mean ± S.E.M.) at the time of sacrifice in peripubertal male and female rats treated neonatally with a leptin antagonist (Antag) or vehicle (Co). n = 6–11 per experimental group. One way ANOVA: *p < 0.05, **p < 0.01,***p < 0.001.

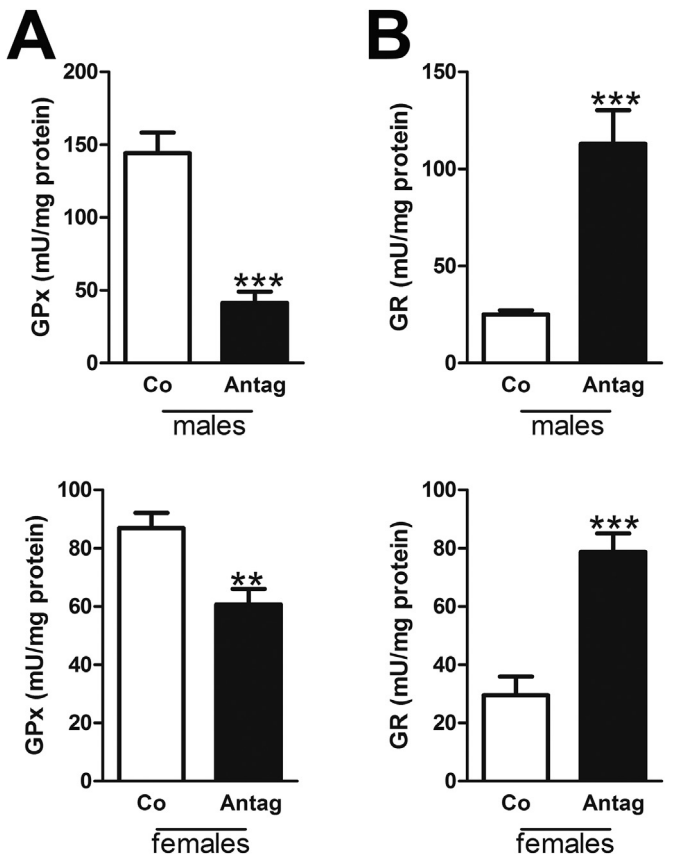


Fig. 4. Changes in GPx (A) and GR (B) activity in spleen homogenate (data expressed as mean ± S.E.M.) at the time of sacrifice in peripubertal male and female rats treated neonatally with a leptin antagonist (Antag) or vehicle (Co). n = 7–12 per experimental group. One way ANOVA: **p < 0.01,***p < 0.001.

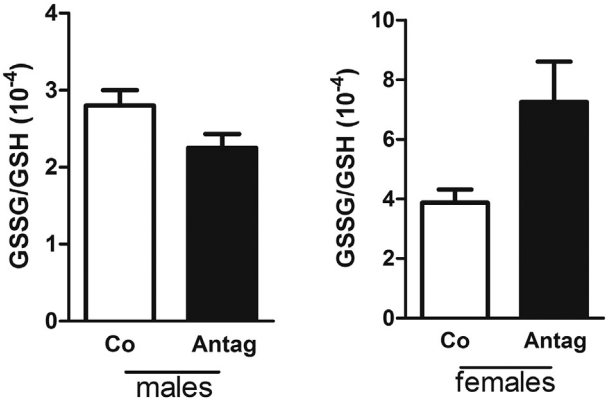


Fig. 5. Changes in GSSG/GSH ratio in spleen homogenate (data expressed as mean ± S.E.M.) at the time of sacrifice in peripubertal male and female rats treated neonatally with a leptin antagonist (Antag) or vehicle (Co). n = 10–12 per experimental group. One way ANOVA: *p < 0.05.

et al., 2014; Valleau and Sullivan, 2014). However, the implication of the neonatal leptin surge in the development and establishment of these responses later in life has not been previously analyzed. In fact, to the best of our knowledge, we provide here the first evidence that disruption of leptin signaling during the time period of the physiological neonatal leptin surge has delayed consequences affecting the oxidative and inflammatory state in peripubertal/adolescent male and female rats.

The spleen is the largest secondary immune organ in the body and is responsible for initiating immune reactions to blood-borne antigens and for filtering the blood of foreign material and old or damaged red blood cells. In rats, this organ reaches peak development at puberty, followed by gradual involution. It appears to be a key player in cytokine production when there is an infection or after trauma resulting in systemic inflammation regulated by the autonomic nervous system (Gigliotti and Okusa, 2014). In this study, we show changes in the spleen weights of peripubertal animals treated neonatally with the leptin antagonist. The spleen of treated males weighed less and those of treated females weighed more than their controls. Since leptin acts as an immunomodulator (Conde et al., 2014; Valleau and Sullivan, 2014), it seems plausible that the treatment affected the correct development of the spleen. Neonatal leptin antagonist treatment clearly impairs the maturation of peripheral tissue such as pancreas, kidney, thymus and ovary in rats (Attig et al., 2011), so it is not surprising to find changes in spleen weight. In addition, we found changes in the weights of adipose tissue and gonads (Mela et al., 2015). Regarding body weight, we found no effect of the leptin antagonist treatment on body weight of either sex during neonatal and prepubertal life, as previously reported (Attig et al., 2008; Granado et al., 2011; Mela et al., 2012a,b). As previously suggested (Mela et al., 2015), one possible explanation for no change in body weight, but a decrease in adipose tissue in males could be that there is an increase in lean body mass.

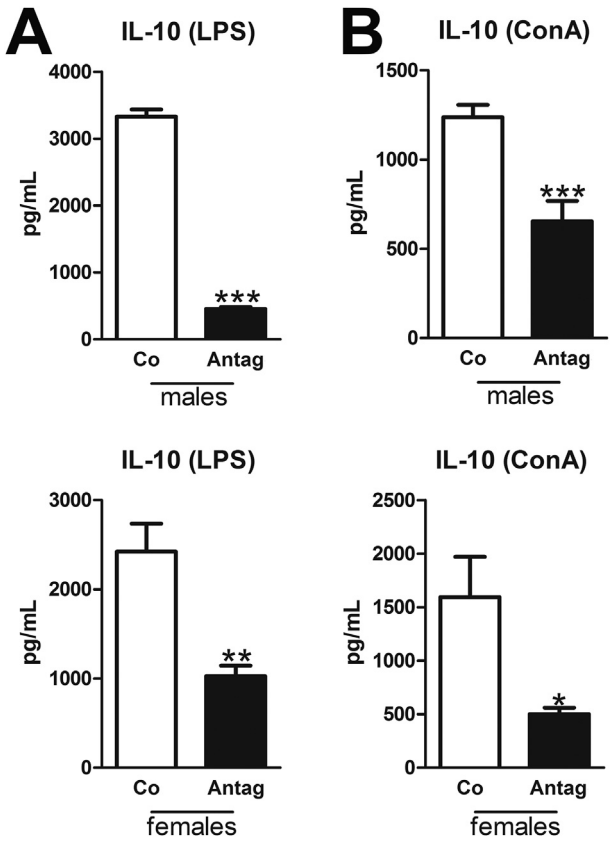


Fig. 6. Changes in IL10 levels released (data expressed as mean ± S.E.M.) by spleen leucocytes treated with either LPS (A) or ConA (B) at the time of sacrifice in peri-pubertal male and female rats treated neonatally with a leptin antagonist (Antag) or vehicle (Co). n = 6–8 per experimental group. ANOVA one way:*p < 0.05, **p < 0.01, ***p < 0.001.

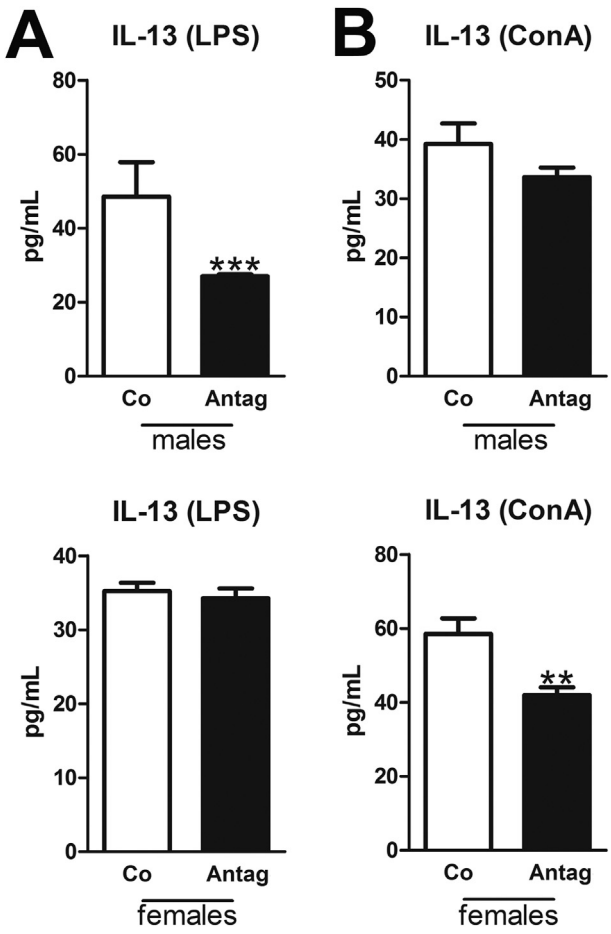


Fig. 7. Changes in IL-13 levels released (data expressed as mean ± S.E.M.) by spleen leucocytes treated with either LPS (A) or ConA (B) at the time of sacrifice in peri-pubertal male and female rats treated neonatally with a leptin antagonist (Antag) or vehicle (Co). n = 7–8 per experimental group. One way ANOVA: **p < 0.01, ***p < 0.001.

SOD acts as an enzymatic antioxidant defense that transforms the superoxide anion to H_2O_2 and works in parallel with CAT, which is responsible for the elimination of the excess of H_2O_2 (Vida et al., 2014). In the present study, we show lower activities of both enzymes in the animals treated in the neonatal period with the leptin antagonist. These results are indicative of decreased antioxidant defenses in the spleen. The glutathione system includes GPx and GR, two enzymes that play an important role in maintaining the correct balance between GSH and GSSG levels. In normal conditions, glutathione is usually found in its reduced form since GR is constitutively active (Vida et al., 2014). In the present study, the treated animals showed higher GR activity and lower GPx activity than the corresponding controls. It is plausible that an increase in GR activity acts as a compensatory mechanism to mitigate the oxidative stress. In fact, when the ratio GSSG/GSH was analyzed as a marker of oxidative stress, no significant effect of the neonatal treatment was found.

Appropriate levels of leptin are needed for a correct immune response (Stofkova, 2009). Leptin appears to stimulate both innate immunity, through the up-regulation of TLR expression in monocytes, and adaptive immune response carried out by lymphocytes. This stimulation is related with the protection against infections and associated with the production of pro-inflammatory cytokines and autoimmune diseases (Conde et al., 2014; Zabeau et al., 2014; Zarkesh-Esfahani et al., 2001). Indeed, both obese ob/ob (leptin deficient) and db/db (deficient in leptin receptors) mice have deteriorated immune responses and increased infection susceptibility (Milner and Beck, 2012) and leptin administration counteracts this impaired immune response in animal models of leptin deficiency (Busso et al., 2002; Lord et al., 1998). In addition, leptin deficiency is associated with decreased production of several cytokines. However, leptin regulation of cytokines is very different depending on several factors, notably the type of stimulus involved (R Faggioni et al., 2000a,b). Therefore, in order to measure the effects of the leptin antagonist on pro-inflammatory and anti-inflammatory cytokine secretion by spleen leukocytes, these cells were stimulated with either LPS or ConA. With both types of stimulation, we found lower IL10 levels in spleen leukocytes from males and females that had been treated with the antagonist. Since IL10 is clearly an anti-inflammatory cytokine, this observation suggests decreased protection against a potential inflammatory state. Moreover, lower levels of anti-inflammatory cytokines such as IL-10 have been detected in ob/ob mice after LPS treatment (Faggioni et al., 2000a,b). The levels of IL13, a typical Th2 cytokine with anti-inflammatory and immunoregulatory actions (Huang et al., 2015; Wynn, 2015), were lower in LPS-stimulated leukocytes from males and in ConA-stimulated leukocytes from females. The pro-inflammatory cytokine TNFα was lower in the LPS-stimulated leukocytes from males treated with the leptin antagonist, whereas the opposite was found in LPS-stimulated leukocytes from treated females, *i.e.* TNFα levels increased. In regards to this latter result, leptin has been shown to inhibit TNFα induction by LPS in female mice (Faggioni et al., 2000a,b). Some results suggest that a

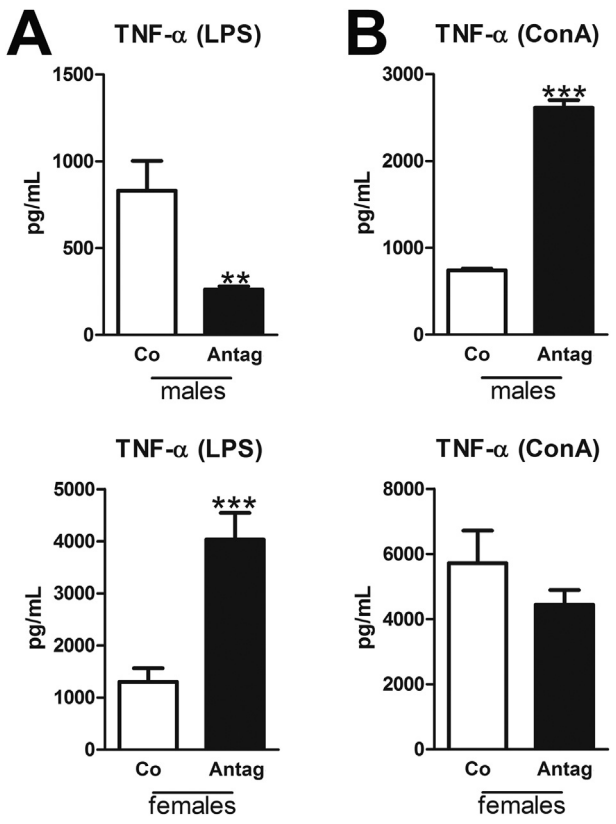


Fig. 8. Changes in TNFα levels released (data expressed as mean ± S.E.M.) by spleen leucocytes treated with either LPS (A) or ConA (B) at the time of sacrifice in peri-pubertal male and female rats treated neonatally with a leptin antagonist (Antag) or vehicle (Co). n = 6–8 per experimental group. One way ANOVA: *p < 0.05, **p < 0.01, ***p < 0.001.

defect in leptin production is associated with a shift of the immune response toward a pro-inflammatory phenotype as consequence of the up-regulation of pro-inflammatory and down-regulation of anti-inflammatory cytokines. However, no conclusive data are available on TNF levels with LPS, with unchanged, decreased or increased production of this cytokine having been reported (Faggioni et al., 2001). As a whole, the present data support the view that the pattern of cytokines regulated by leptin is very different depending on the type of stimulus used to induce their release (R Faggioni et al., 2000a,b). Moreover, we show clear differences between males and females. It is worth re-emphasizing that, due to the reason indicated in the method section, the males and females used in this study were of different chronological ages and, therefore, the differences observed between males and females may not be exclusively attributed to their sex. This does not negate the fact that blockage of neonatal leptin signaling affects the future oxidative status of the animals and their ability to respond to inflammatory insults during the critical period close to pubertal onset in both males and females.

When we analyzed if there were changes in the immune system in other tissues, we found significant effects in WAT. Males appeared to be more affected by the treatment with leptin antagonist than females, as they presented an increase in IL1b and IL6 and a decrease in CAT and GR mRNA levels. However, we found a decrease in TNFa mRNA levels in females treated with the antagonist, corroborating the idea that the effect of this leptin antagonist is sex dependent. How these effects compromise the health status of individuals in the presence of an injury or immune challenge is unknown. Further investigation is needed to clarify the inflammatory state of these animals throughout their life.

We have previously found that maternal deprivation (MD) during 24 h on postnatal day 9 (*i.e.*, during the neonatal leptin surge period) produces a marked decrease in leptin levels (Viveros et al., 2010), as well as short- and long-term detrimental effects on the immune system (De La Fuente et al., 2009; Viveros et al., 2009). Thus, it is likely that both a social intervention such as MD and the present pharmacological treatment affect the development of the organism's defense system by interfering with the neonatal leptin surge.

5. Conclusion

These findings point to a possible programming effect of the neonatal leptin surge on the establishment of an appropriate redox and inflammatory state. Since this is relevant to maintain a healthy state in adulthood and throughout the aging process (De la Fuente and Miquel, 2009; Hunsche et al., 2015), it follows that any situation that avoids/interferes with the neonatal leptin surge could represent a risk factor for the future health of the individual.

Table 1
Hypothalamic mRNA levels.

		IL-10	TNFα	CAT	SOD	GPx	GR
Males	Co	100 ± 19.3	100 ± 7.6	100 ± 5.3	100 ± 3	100 ± 3	100 ± 5
	Antag	76.5 ± 23.2	152.8 ± 26.8	106.9 ± 4	105.6 ± 1.7	112.6 ± 5.7	107.4 ± 3.7
Females	Co	100 ± 17.5	100 ± 6.2	100 ± 12	100 ± 7.8	100 ± 9.8	100 ± 4.3
	Antag	76.4 ± 16.6	75.2 ± 7.3*	88.3 ± 6.2	87.3 ± 3	108.8 ± 4.7	101.8 ± 4.1

Data are expressed as mean ± SEM from rats treated with vehicle (Vh) or with leptin antagonist treatment (Antag) from postnatal day (PND) 5 to PND9. Data are normalized to Co treated rats for each sex. N = 4–6 per experimental group. One-Way ANOVA: *p < 0.05.

Table 2
WAT mRNA levels.

		IL-1β	IL-6	CAT	SOD	GPx	GR
Males	Co	100 ± 4.4	100 ± 13	100 ± 18.4	100 ± 11.6	100 ± 13.6	100 ± 18.1
	Antag	262.9 ± 63.7**	237.4 ± 25.6***	47.3 ± 15.3*	67.7 ± 14.9	68.3 ± 14.6	50.4 ± 28.3*
Females	Co	100 ± 12.42	100 ± 44	100 ± 18.7	100 ± 22.8	100 ± 26.4	100 ± 24
	Antag	149.7 ± 45.5	62.1 ± 19.3	62.6 ± 14.3	58.23 ± 9.3	78.1 ± 15	78 ± 15.4

Data are expressed as mean ± SEM from rats treated with vehicle (Vh) or with leptin antagonist treatment (Antag) from postnatal day (PND) 5 to PND9. Data are normalized to Co treated rats for each sex. N = 4–6 per experimental group. One-Way ANOVA: *p < 0.05, **p < 0.01, ***p < 0.005.

Acknowledgements

This work was supported by the Ministerio de Ciencia e Innovación Grants BFU2012-38144, BFU2011-30336 and BFU2014-51836-C2-2; Research groups of UCM (951579 and 910379), Redes temáticas de Investigación Cooperativa en Salud RD2012/0028/0021 and RD12/0043/0018, as well as FIS (PI15/01787) from the ISCIII-FEDER of the European Union.

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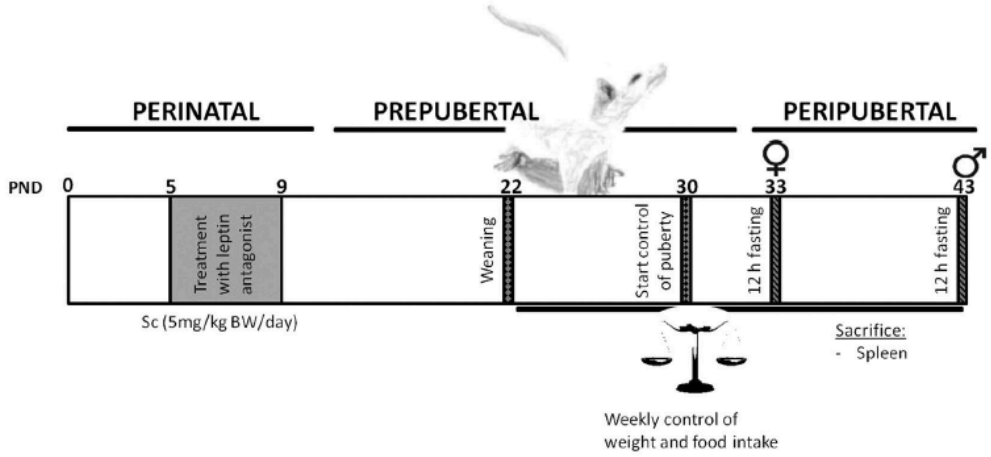
3.2.2. Effects of the blockage of the neonatal leptin surge (PND5-9) on immune function and inflammatory state in spleen leukocytes of peripubertal/adolescent male and female rats.



Experimental design

The animals used in this study were the same as those used in the previous sub-objective that focused on the effects of the leptin antagonist treatment on the redox/inflammatory state of the spleen, hypothalamus and white adipose tissue in peripubertal/adolescent rats.

From PND5 until PND9 rats were injected with 5 mg/kg bodyweight (bw) of rat pegylated super leptin antagonist (mutant D23L/L39A/D40A/F41A). The animals received one subcutaneous injection on their back per day at 9:00 am. In order to avoid the stress effect caused by marking the animals at this early age, each litter was treated with either vehicle (controls) or antagonist. Control rats were injected with the same volume of distilled water as the vehicle (2.5 ml/kg). After each injection the animals were immediately returned to their mothers. Each experimental group consisted of 12 animals. To avoid/minimize possible litter effects, all experimental groups contained animals from at least three different litters. Female (PND33) and male (PND43) rats were sacrificed after a 12 h fast by rapid decapitation. The spleen was rapidly and aseptically removed. The spleen was then freed of fat and divided into two parts. One fragment of the spleen was stored at -80 °C for the study of the redox parameters (data from the previous sub-objective). The other fragment of the spleen was minced with scissors, and gently pressed through a mesh screen to

obtain the cell suspension. These cell suspensions were centrifuged in a gradient of Ficoll-Hypaque and used in the present sub-objective to evaluate immune function and inflammatory state of spleen leukocytes in peripubertal/adolesecent rats.

Main results

Spleen leukocyte function parameters

Peripubertal male and female rats exposed to the leptin antagonist in the neonatal period showed in spleen leukocytes significantly lower values of anti-tumour NK activity than those in cells of controls. The chemotaxis capacity of spleen leukocytes also showed lower values in treated male and female rats than in controls. The lymphoproliferation in response to T-cell mitogen (ConA) and B-cell mitogen (LPS) was significantly lower in female treated rats than in controls. However, no differences were found in the lymphoproliferation of male rats with respect to controls.

Cytokine levels

With respect to the cytokine IL-2 released in response to ConA and LPS-stimulated leukocytes, the concentrations were lower in treated females than in controls. In males, the concentrations of IL-2 in ConA-stimulated leukocytes were higher than in controls, but no differences were observed in cultures with LPS.

The values of IL-1α and IL-1β were lower, in general, in peripubertal male and female rats treated neonatally with the leptin antagonist than in controls. The differences were statistically significant in the case of females for IL-1α with ConA and LPS as well as for IL-1β with ConA. In males statistical significances were observed for IL-1α and IL-1β in presence of LPS.

The values of IL-6 were lower in treated animals than in the corresponding controls, in LPS-stimulated leukocytes from males, as well as in ConA and LPS-stimulated leukocytes from females.

The IL-12p70 concentrations were lower in leukocytes from treated females in the presence of ConA and in those from treated males in the presence of LPS, than in controls. However, IFN-γ values were higher in male treated rats under ConA-stimulated conditions than in controls.

The GM-CSF showed lower levels in male treated rats under LPS-stimulated conditions than in controls. Peripubertal female rats showed a tendency for lower levels in ConA-stimulated spleen leukocytes with respect to controls.

The Th2 cytokines, IL-4 and IL-5, which are commonly known for their anti-inflammatory actions, showed lower values in leukocytes from treated animals than in controls. The differences were statistically significant only in the case of males, in cultures in the presence of LPS.

Partial conclusions

The results of this experiment indicate that the blockage of the neonatal leptin surge results in an impairment of immune function and in an altered cytokine release of spleen leukocytes in peripubertal male and female rats.

Fourth article regarding sub-objective 2.b.

Manuscript Details

Manuscript number	DCI_2019_209
Title	BLOCKAGE OF POSTNATAL LEPTIN SURGE IMPAIRS SPLEEN LEUKOCYTE FUNCTIONS IN PERIPUBERTAL RATS
Article type	Full Length Article

Abstract

Leptin is a pleiotropic hormone with not only an important role in the central regulation of energy homeostasis, but also involved in other peripheral processes, such as the modulation of immune system functions. There is a physiological surge of leptin in rodents during the neonatal period, which has been mainly studied in relation to its neurotropic role during brain development. However, little is known about the effects of this neonatal leptin surge in relation to immunity. Therefore, we investigated if the blockage of this leptin surge could affect several immune functions. A leptin antagonist administered during the neonatal period (PND5-10) in male and female rats were used, and in the peripubertal period of these animals relevant functions as well as cytokine release by spleen leukocytes were studied. The results showed that these animals displayed significantly impaired anti-tumor NK activity, chemotaxis capacity and proliferation of lymphocytes in response to mitogens. In addition, several cytokine concentrations, released under mitogen-stimulated conditions, were also altered. In conclusion, the neonatal leptin surge seems to be involved in the establishment of an adequate immune response and cytokine profile, which are crucial for the maintenance of a healthy life.

Keywords	Neonatal leptin; Leptin antagonist; Immune function; Cytokine release; Peripubertal rats
Taxonomy	Molecular Biology, Biochemistry
Manuscript category	Vertebrate

Corresponding Author	Monica De la Fuente
Order of Authors	Caroline Hunsche, Oskarina Hernandez, Virginia Mela, Maria Paz Viveros, Monica De la Fuente

Suggested reviewers	VICTOR SANCHEZ-MARGALET, Gilberto Paz-Filho, Oreste Gualillo

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Research Data Related to this Submission

There are no linked research data sets for this submission. The following reason is given:
Data will be made available on request

Madrid, May 31th, 2019

Dear Editor-in-Chief,

We are pleased to submit the manuscript entitled "Blockage of leptin surge impairs spleen leukocytes functions in peripubertal rats", for its possible publication in Developmental & Comparative Immunology.

This is an original study focused on the effects that the exposure to a leptin antagonist during the neonatal period could have on immune function and cytokine release in spleen leukocytes of peripubertal male and female rats.

This paper should be of interest to readers of Developmental & Comparative Immunology since it provides evidence that that the blockage of the neonatal leptin surge leads to impaired immune and inflammatory functions in the peripubertal period. Therefore, the neonatal leptin surge seems to have an important physiological role in the establishment and maintenance of adequate immune and inflammatory responses.

We state that the data presented in the manuscript have not been published elsewhere, in whole or in part, and that this work is not currently under review or accepted for publication in other journals. All authors have contributed to the final manuscript and all authors have read, approved and agreed to submit the manuscript to Developmental & Comparative Immunology.

Yours sincerely,

Mónica De la Fuente

Department of Genetic, Physiology and Microbiology. Faculty of Biology, Complutense University of Madrid

José Antonio Novais Street, 12. 28040. Madrid. Spain.

Telephone number: 0034 91 3944989

Fax number: 0034 91 3944935

Email address: mondelaf@bio.ucm.es

Highlights

- Neonatal blockage of leptin affects the immune response of peripubertal rats.
- Neonatal blockage of leptin affects the release of cytokines of peripubertal rats.
- Potential role of neonatal leptin surge in the development of immune functions.

BLOCKAGE OF POSTNATAL LEPTIN SURGE IMPAIRS SPLEEN LEUKOCYTE FUNCTIONS IN PERIPUBERTAL RATS

Caroline Hunsche[1,2], Oskarina Hernandez[1], Virginia Mela[1], M. Paz Viveros[1], Mónica De la Fuente[1,2]

[1]Department of Genetics, Physiology and Microbiology (Unit of Animal Physiology). Faculty of Biology, Complutense University of Madrid.

José Antonio Novais Street, 12. 28040. Madrid. Spain.

[2]Research Institute of Hospital 12 de Octubre (i+12).

***Corresponding author:**

Mónica De la Fuente

Department of Genetic, Physiology and Microbiology. Faculty of Biology, Complutense University of Madrid

José Antonio Novais Street, 12. 28040. Madrid. Spain.

Telephone number: 0034 91 3944989

Fax number: 0034 91 3944935

Email address: mondelaf@bio.ucm.es

Abstract

Leptin is a pleiotropic hormone with not only an important role in the central regulation of energy homeostasis, but also involved in other peripheral processes, such as the modulation of immune system functions. There is a physiological surge of leptin in rodents during the neonatal period, which has been mainly studied in relation to its neurotropic role during brain development. However, little is known about the effects of this neonatal leptin surge in relation to immunity. Therefore, we investigated if the blockage of this leptin surge could affect several immune functions. A leptin antagonist administered during the neonatal period (PND5-10) in male and female rats were used, and in the peripubertal period of these animals relevant functions as well as cytokine release by spleen leukocytes were studied. The results showed that these animals displayed significantly impaired anti-tumor NK activity, chemotaxis capacity and proliferation of lymphocytes in response to mitogens. In addition, several cytokine concentrations, released under mitogen-stimulated conditions, were also altered. In conclusion, the neonatal leptin surge seems to be involved in the establishment of an adequate immune response and cytokine profile, which are crucial for the maintenance of a healthy life.

Keywords: Neonatal leptin; Leptin antagonist; Immune function; Cytokine release; Peripubertal rats

1. Introduction

Leptin is a 16-kDa pleiotropic hormone with not only an important role in the central regulation of energy homeostasis, but also involved in other peripheral processes, such as the modulation of immune system functions (Feng et al., 2013; Lago et al., 2008). Leptin is predominantly secreted by adipocytes in adipose tissue but at lower levels also by skeletal muscle, stomach and placenta (Bado et al., 1998; Sagawa et al., 2002; Señarís et al., 1997; Wang et al., 1998). Increasing evidence suggests that leptin has an important role in fetal development. For instance, in pregnancy, the production in the placenta rises significantly in rodents and humans (Gavrilova et al., 1997; Sagawa et al., 2002; Señarís et al., 1997). In addition, there is a temporary increase of leptin levels in rodents during the early postnatal period, constituting a "leptin surge". Thus, it has been described that neonatal mice and rats experience elevated levels of leptin from postnatal day (PND) 4 to PND 14, with a peak at PND 10 (Ahima et al., 1998; Delahaye et al., 2008). This leptin surge, which is not influenced by nutritional intake and body fat (Ahima et al., 1998), seems to be important for brain development, particularly in the establishment of hypothalamic circuits that regulate food intake (Bouret et al., 2004). Currently, it is well known that early life is a critical developmental period and stresses generated in this period could result in long-lasting detrimental effects in later life (De la Fuente et al., 2009; Lucassen et al., 2013; Viveros et al., 2009). Indeed, perturbations in the early neonatal circulation levels of leptin in rodents have been associated with long-terms alterations in energy homeostasis and reproduction (Attig et al., 2008; Mela et al., 2015).

Although leptin receptors are highly expressed in the central nervous system, they can also be detected in other systems, such as the immune system (Lord et al., 1998; Mercer et al., 1996). The expression of leptin receptors in immune system cells (including macrophages, monocytes, neutrophils, natural killer (NK) cells, T and B cells), which showed to be increased by leptin stimulation, indicates that leptin may play an important role in the regulation of immune and inflammatory responses (Naylor and Petri, 2016; Zhao et al., 2003). In fact, nutritional dysfunctional states, such as malnutrition and obesity, in which there are diminished or enhanced levels of leptin, respectively, have been shown to lead to a poorer immune response against infection (Zhang et al., 2005). Thus, increasing evidence suggests that adequate levels of leptin are essential for the maintenance of an optimal immune response. Leptin, which shares similarities with members of the cytokine family (Zhang et al., 2005), has been shown to also function as an inflammatory molecule. It has been reported that leptin is able to modulate a variety of immune system functions, such as chemotaxis, NK cell activity, proliferation of lymphocytes and the production of inflammatory cytokines (Gainsford et al., 1996; Gruen et al., 2007; Lago et al., 2008; Lam and Lu, 2007; Lord, 2006; Lord et al., 1998; Martín-Romero et al., 2000; Naylor and Petri, 2016; Santos-Alvarez et al., 1999; Tian et al., 2002; Zhang et al., 2005; Zhao et al., 2003).

Preliminary research suggests an association between the neonatal leptin surge and the immune system. For instance, leptin blockage during the early neonatal phase affected the maturation of key immune organs, such as thymus

and spleen (Attig et al., 2011). Also, we have recently reported that the exposure to a leptin antagonist during the postnatal period resulted in alterations of redox and inflammatory state in spleen, hypothalamus and adipose tissue of peripubertal male and female rats (Mela et al., 2017). Nevertheless, nothing is known about the physiological role of the postnatal leptin surge in functions of the immune system. Therefore, the aim of the present study was to determine if the use of a leptin antagonist during the period corresponding to the physiological surge of leptin (PND5-10) could affect relevant functions of the immune cells as well as cytokine release by spleen leukocytes in peripubertal male and female rats.

2. Materials and methods

2.1. Animals

Experimental subjects were the offspring of Wistar rats purchased from Harlan Interfauna Ibérica S.A. (Barcelona, Spain). The parental generation was mated (one male x two females) in our animal facilities approximately two weeks after their arrival. After 10 days, female animals were isolated and the day of delivery strictly controlled. On the day of birth (postnatal day 0, PND0), litters were sex-balanced and culled to eight pups per dam (four males and four females). No cross-fostering was employed. Thus, only litters with at least four pups of each sex at birth were used. The animals were housed in plastic Makrolon® III cages and maintained at a constant temperature (22 ± 1°C) and humidity (50 ± 2%) in a reversed 12-h light-dark cycle (red light on at 8:00 am and white light on at 8:00 pm). Pregnant rats were given free access to food (commercial diet for rodents; A03, Safe, Augy, France) and water. The animals used in this study were the same as those used in a recently published paper that focused on the effects of the same leptin antagonist treatment on hypothalamic systems related to reproduction (Mela et al., 2015). As the aim in that study was to investigate the pubertal transition, immediately before the appearance of external phenotypic signs of puberty, and because the timing of puberty differs between the sexes, females were euthanized on PND33 and males on PND43. These studies were approved by the local ethics committee and complied with Royal Decree 1201/2005 (BOE n° 252) pertaining to the protection of experimental animals and with the European Communities Council Directive (86/609/EEC). The experiments were conducted in accordance with the guidelines and protocols of Royal Decree 53/2013 regarding the care and use of laboratory animals for experimental procedures, and were approved by the Committee for Animal Experimentation of the Complutense University of Madrid.

2.2. Leptin antagonist treatment

From PND5 until PND9 rats were injected with 5 mg/kg bodyweight (bw) of rat pegylated super leptin antagonist (mutant D23L/L39A/D40A/F41A), a gift of Protein Laboratories (Rehovot, Israel), as previously described (Jamroz-Wiśniewska et al., 2014). The animals received one subcutaneous injection on their back per day at 9:00 am. In order to avoid the stress effect caused by marking the animals at this early age, we treated each litter with either vehicle (controls) or antagonist. Control rats were injected with the same volume of distilled water as the vehicle (2.5 ml/kg). After each injection the animals were immediately returned to their mothers. Each experimental group consisted of 12 animals. To avoid/minimize possible litter effects all experimental groups contained animals from at least three different litters.

2.3. Collection of spleen and leukocyte suspensions

Female (PND33) and male (PND43) rats were sacrificed after a 12 h fast by rapid decapitation. The spleen was rapidly and aseptically removed. The spleen was then freed of fat and divided into two parts. One fragment of spleen was stored at -80 °C for the study of the oxidative stress parameters (data previously published) (Mela et al., 2017). Another fragment of spleen was minced with scissors, and gently pressed through a mesh screen (Sigma-Aldrich, Madrid, Spain) to obtain the cell suspension. The cell suspensions were centrifuged in a gradient of Ficoll-Hypaque (Sigma-Aldrich) with a density of 1.070 g/ml. Cells from the interface were collected and suspended in Roswell Park Memorial Institute (RPMI) 1640 medium enriched with L-glutamine (PAA, Pasching, Austria) and supplemented with 10% heat-inactivated (56 °C, 30 min) fetal calf serum (PAA) and gentamicin (100 µg/ml, PAA). After a wash step, leukocytes were counted in a Neubauer chamber (Blau Brand, Wertheim, Germany) and their number adjusted to 10^6 cells/ml. Cell viability was routinely measured before each experiment by the trypan-blue exclusion test, and was higher than 98% in all experiments. All incubations were performed at 37 °C in a humidified atmosphere of 5% CO_2.

2.4. Immune functions

2.4.1. NK activity assay

An enzymatic colorimetric assay was carried out to measure the cytolysis of tumor cells (Cytotox 96 TM Promega, Boeringher Ingelheim, Germany) based on the determination of lactate dehydrogenase enzyme (LDH), as previously described (De la Fuente et al., 2004). Aliquots of 100 µl of leukocytes, used as effector cells, were seeded in 96-well U-bottom culture plates (Numc, Roskilde, Denmark) adjusted to 10^6 leukocytes/ml in RPMI 1640 medium without phenol red. Murine lymphoma YAC-1 cells, used as target cells, were added adjusted to 10^5 cells/ml. Thus, the effector/target

ratio was 10:1. The plates were centrifuged at 250 g for 4 min to facilitate cell contacts. After 4 hours of incubation, lactate dehydrogenase enzymatic activity was measured in 50 µl/well of the supernatants by addition of the enzyme substrate and absorbance recording spectrophotometrically at 490 nm. Three kinds of control measurements were performed: a target spontaneous release, a target maximum release, and an effector spontaneous release. The results were expressed as percentages of lysis of target cells. To determine this percentage the following equation was used: %lysis = ((E-ES-TS)/M-ES-TS)) x 100, where E is the mean of absorbance in the presence of effector cells; ES, the mean of absorbance of effector cells incubated alone; TS, the mean of absorbance in target cells incubated with medium alone; and M is the mean of maximum absorbance after incubating target cells with lysis solution.

2.4.2. Chemotaxis assay

Chemotaxis of leukocytes was evaluated according to a slight modification of Boyden´s method (De la Fuente et al., 2004) consisting basically of the use of chambers with two compartments separated by a filter with a pore diameter of 3 µm (Millipore, Bedford, MA, USA). Aliquots of 300 µl of the leukocyte suspensions, adjusted to $5X10^5$ cells/ml in Hank´s solution, were deposited in the upper compartment, and aliquots of 400 µl of the chemoattractant, formyl-Met-Leu-Phe (10^{-8} M) (Sigma-Aldrich), were put into the lower compartment. The chambers were incubated for 3h and then the filters were fixed and stained. The chemotaxis index (CI) was determined by counting, in an optical microscope (100X), the total number of lymphocytes on one third of the lower face of the filters.

2.4.3. Lymphoproliferation assay

The proliferation of lymphocytes in response to the mitogens concanavalin A (ConA) and lipopolysaccharide (LPS) was measured following a method previously described (De la Fuente et al., 2004). Aliquots (200 µl) of spleen leukocytes (10^6 cells/ml complete medium) were seeded in 96 well flat-bottomed microtiter plates (Numc, Roskilde, Denmark), and 20 µl of ConA (1µg/ml; Sigma-Aldrich), a T-cell mitogen (lectin) or 20 µl of lipopolysaccharide (*Escherichia coli*, 055:B5 1µg/ml; Sigma-Aldrich), a B-cell mitogen, were added per well. After 48h of incubation at 37°C in an atmosphere of 5% CO2, 0.5 µCi ^{3}H-thymidine (Du Pont, Boston, MA, USA) were added to each well. The cells were harvested in a semiautomatic microharvester 24h later. Thymidine uptake was measured using a beta counter (LKB, Uppsala, Sweden) and the results were expressed as ^{3}H-thymidine uptake (cpm).

2.5. Cytokine concentrations

The concentrations of the cytokines, including interleukin-2 (IL-2), interleukin-1α (IL-1α), interleukin-1β (IL-1β), interleukin-6 (IL-6), interleukin-12p70 (IL-12p70), interferon-γ (IFN-γ), granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin IL-4 (IL-4) and interleukin-5 (IL-5), released into the supernatant of spleen leukocytes cultures, after 48 hours of incubation with ConA or LPS, were measured simultaneously using a Luminex xPONENT (Rat Bio Plex Pro Cytokine assays using magnetic beads, Bio-Rad, USA). The results were expressed as pg/mL.

2.6. Statistical analysis

SPSS 21.0 (SPSS, Inc., Chicago, USA) was used for the statistical analysis of the results. The data were expressed as mean ± standard deviation (SD). Each value is the mean of the data from an assay performed in duplicate or triplicate. Normality of the samples was checked by the Kolmogorov-Smirnov test and homogeneity of variances with the Levene test. The data were statistically evaluated by the Student´s *t*-test for independent samples. $P<0.05$ was considered as statistically significant and $0.05<P<0.1$ as a trend. As males and females were of a different chronological age due to the differential timing of the onset of puberty, data were not directly compared between sexes.

3. Results

3.1. Lymphocyte immune functions

The immune functions of spleen leukocytes are shown in Figure 1. Peripubertal male and female rats exposed to the leptin antagonist in the neonatal period showed significantly lower values of activity of NK cells than those in controls ($P<0.001$ and $P<0.05$, respectively. Figures 1C and 1A). The chemotaxis capacity of spleen leukocytes (Figures 1D and 1B) also showed lower values in treated male and female rats than in controls ($P<0.001$).

The lymphoproliferation in response to T-cell mitogen (ConA) and B-cell mitogen (LPS) (Figure 1) was significantly lower in female treated rats than in controls ($P<0.05$. Figures 1A and 1B). However, no differences were found in the lymphoproliferation in male rats with respect to controls (Figures 1C and 1D).

3.2. Cytokine concentrations

The concentrations of several cytokines released in spleen leukocytes cultured in presence of the mitogens ConA or LPS, are shown in Figures 2 to Figure 7. They have important roles regulating innate and adaptive immune functions and belong to the T helper type1 (Th1) or T helper type 2 (Th2) group.

With respect to the cytokine IL-2 released in response to ConA and LPS-stimulated leukocytes (Figure 2), the concentrations were lower in treated females than in controls ($P<0.01$ and $P<0.001$, respectively. Figures 2A and 2B). In males, the concentrations of IL-2 in ConA-stimulated leukocytes were higher than in controls ($P<0.05$), but no differences were observed in cultures with LPS (Figures 2C and 2D).

The values of IL-1α and IL-1β concentrations (Figure 3) were lower, in general, in peripubertal male and female rats treated neonatally with the leptin antagonist than in controls. The differences were statistically significant in the case of females for IL-1α with ConA and LPS as well as for IL-1β with ConA ($P<0.05$. Figures 3A, 3B and 3E). In males statistical significances were observed for IL-1α and IL-1β in presence of LPS ($P<0.05$ and $P<0.01$, respectively. Figures 3D and 3H).

The values of IL-6 were lower in treated animals than in the corresponding controls, in LPS-stimulated leukocytes from males ($P<0.001$. Figure 4D) as well as in ConA and LPS-stimulated leukocytes from females ($P<0.05$. Figures 4A and 4B).

The IL-12p70 concentrations were lower in leukocytes from treated females in the presence of ConA and in those from treated males in the presence of LPS, than in controls ($P<0.05$. Figures 5A and 5D, respectively). However, IFN-γ values were higher in male treated rats under ConA-stimulated conditions than in controls ($P<0.01$. Figure 5G).

The GM-CSF showed lower concentrations in male treated rats under LPS-stimulated conditions than in controls ($P<0.05$. Figure 6D). Peripubertal female rats showed a tendency for lower concentrations in ConA-stimulated spleen leukocytes with respect to controls ($0.05<P<0.1$. Figure 6A).

The Th2 cytokines, IL-4 and IL-5, which are commonly known for their anti-inflammatory actions, showed lower values in leukocytes from treated animals than in controls (Figure 7). The differences were statistically significant only in the case of males, in cultures in the presence of LPS ($P<0.05$. Figures 7D and 7H).

4. Discussion

In the present work, we demonstrated for the first time that the exposure to a leptin antagonist during a critical developmental period of early postnatal life resulted in impaired immune function and cytokine release in spleen leukocytes of peripubertal male and female rats. We carried out the study in spleen since this secondary lymphoid organ, which is the largest in both humans and rodents, is responsible for mounting effective innate and adaptive immune responses and for regulating cytokine production (Nolte et al., 2002; Cesta, 2006; Huston et al., 2006). The animals treated with a specific leptin antagonist during the leptin surge (PND5-10) displayed significantly impaired chemotactic capacity, anti-tumor NK activity and proliferation of lymphocytes in response to mitogens (LPS and

ConA). Moreover, in cultures of those leukocytes under ConA and LPS-stimulated conditions, an impaired of the release of several cytokines was shown in general in these rats treated with the leptin antagonist.

The immune system in early life goes through rapid and critical changes. Thus, perturbations in this period could result in long-lasting detrimental effects in later life (Goenka and Kollmann, 2015). In this sense, the neonatal programming of adult immune function has been previously reported. Thus, the neonatal exposure to an immune challenge, such as bacterial endotoxin LPS, showed to alter neuroendocrine and immune responses in adult animals (Spencer et al., 2001; Skanks et al., 1995; Shanks et al., 2000). Several mechanisms underlying these effects seem to be related to hypothalamic-pituitary-adrenal axis modifications (Spencer et al., 2001). In fact, a previous study in which rats were neonatally treated with the same leptin antagonist found long-term alterations on hypothalamic expression of reproductive and metabolic neuropeptides (Mela et al., 2016). Given the bidirectional communication between nervous and immune system, this alteration exerted on the hypothalamus could also have an impact on the immune system (Vida et al., 2014). In addition, although there is increasing evidence showing the effects of leptin on several immune system functions, no information is available specifically correlating the neonatal leptin surge with these functions at peripubertal age. For this, in the present study, the pegylated super leptin antagonist was administrated in male and female rats from PND5 to 9. This period of time is coincident, according with previous reports, to the plasma surge of leptin in neonatal mice and rats (Ahima et al., 1998; Delahaye et al., 2008). With respect to the innate immunity, the functions studied were significantly impaired in spleen leukocytes of peripubertal male and female rats neonatally treated with leptin antagonist. Thus, the NK cells showed a lower lytic activity against tumor cells in treated animals than in controls. Therefore, it seems that the leptin surge has an important role in establishing and maintaining the proper function of NK cells. In fact, it was previously reported that leptin is involved in the processes of NK cell development, differentiation, activation, and cytoxicity (Tian et al., 2002; Zhao et al., 2003). It was found that leptin enhanced these processes in NK cells via the activator of transcription 3 (STAT-3) and by the up-regulation of the expression of IL-2 and perforin genes (Zhao et al., 2003). In relation to the chemotaxis activity, this function was suppressed in cells from male and female treated animals. The role of leptin in the migration of immune cells has been observed given that it shows a chemoattractant capacity at low concentrations. This effect seems to be mediated via full-length leptin receptors and canonical migratory signaling pathways (Gruen et al., 2007). Moreover, leptin has been shown to stimulate the expression of chemokines in murine macrophages through the activation of the janus kinase 2 (JAK-2)-signal transducer and STAT3 pathway (Kiguchi et al., 2009).

The neonatal administration of the leptin antagonist also resulted in impaired adaptive immunity in peripubertal rats. Thus, the proliferation of spleen lymphocytes in response to T- and B-cell mitogens, such as ConA and LPS, respectively, was lower in peripubertal female treated rats than in controls. An increased proliferation of lymphocytes has been observed in the presence of leptin (Lord et al., 1998). An enhanced proliferation and activation of human T

lymphocytes with leptin was also observed when cells were co-stimulated with phytohemagglutinin (PHA) or concanavalin A (ConA) (Martín-Romero, 2000). Moreover, leptin also plays an important role in the development of lymphocytes. In this sense, leptin receptor-deficient db/db mice showed impaired lymphopoiesis and reduced numbers of lymphocytes in peripheral blood (Bennett et al., 1996).

Increasing evidence suggests that leptin has a regulatory role within the cytokine network (Gainsford et al., 1996; Santos-Alvarez et al., 1999; Martín-Romero et al., 2000; Lord, 2006; Lam and Lu, 2007). Thus, in the present study, in agreement with the impairments found in innate and adaptive immune functions, the neonatal leptin antagonist treatment also resulted, in general, in a lower release of several cytokines in ConA and LPS-stimulated spleen leukocytes of peripubertal rats. The IL-2, which is an essential regulatory cytokine that acts mainly on lymphoid populations, including T, B, and NK cells (Liao et al., 2011), was found to be diminished in female neonatally treated animals. In this sense, leptin has been shown to influence the proliferation and secretion of IL-2 by immune cells, through the activation of the mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3-kinase (PI3-K) pathways (Liao et al., 2011; Lord et al., 1998). Since IL-2 is related to the proliferation of lymphocytes, the lower concentrations of this cytokine in treated females could explain the reduced lymphoproliferative response in the cells of this sex.

In addition, several studies have shown that leptin signaling increases inflammatory mediators through direct activation of immune cells (Matarese et al., 2005; Procaccini et al., 2012; Spencer et al., 2001; Tian et al., 2002). Thus, IL-1α and IL-1β, which are classic pro-inflammatory cytokines and are also involved in the proliferative response of lymphocytes (Zarkesk-Esfahani et al., 2001), exhibited lower concentrations in peribupertal male and female rats neonatally treated with leptin antagonist under LPS or ConA-stimulated conditions. Lower concentrations of IL-6 were also found in treated peripubertal male and female animals under mitogen (LPS or ConA) stimulation. Together with IL-2, IL-1α and IL-1β, IL-6 is crucial in the orchestration of the differentiation and function of innate and adaptive immune cells (Dinarello, 2009). These cytokines regulate T lymphocyte activation and differentiation (GArlanda et al., 2013; Sims and Smith, 2010). In addition, IL-1β and IL-6 promote B cell functions and antibody production (Dinarello, 2009; Fisher et al., 2014). Previous studies have clearly demonstrated an association between the regulation of these cytokines and leptin. Thus, leptin has been shown to directly induce the release of IL-1β and IL-6 in microglia cells (Ataie-Kachoie et al., 2014; Tang et al., 2007) and to mediate the LPS-induced symptoms of sickness behavior through a process that involves IL-1β induction in the brain (Lafrance et al., 2010; Sachot et al., 2004).

The concentrations of IL-12p70, a pro-inflammatory cytokine known to promote Th1 immune differentiation against intracellular pathogens, including bacteria and some viruses (Raphael et al., 2015), were lower in ConA or LPS-stimulated leukocytes from male and female treated rats. In addition, GM-CSF concentrations were diminished in LPS-stimulated leukocytes from treated males and it should be borne in mind that this cytokine, which has been typically

recognized as a hematopoietic growth factor, plays an important role in Th1 and Th17 responses (Shiomi and Usui, 2015). However, the concentrations of IFN-γ, also known as a Th1 cytokine with pro-inflammatory properties, were increased in ConA-stimulated leukocytes from treated males, whereas no differences were found in female treated rats. A study recently published, in which the same experimental design was used (Mela et al., 2017), also found increased concentrations of TNF-α (which is also a Th1 cytokine with pro-inflammatory properties) in ConA-stimulated leukocytes from treated males. In addition, previous data indicate that, in several cases, a defect in leptin production could lead to an increase in the secretion of several pro-inflammatory cytokines, favoring the up-regulation of pro-inflammatory mediators (Faggioni et al., 2001). However, other studies using rodents with genetic abnormalities in leptin or leptin receptors have shown suppressed expression of pro-inflammatory cytokines, such as IL-12 and IL-6, in response to LPS stimulation (Loffreda et al., 1998).

The release of T-helper type 2 (Th-2) cytokines, including the anti-inflammatory cytokines IL-4 and IL-5, which are commonly involved in enhancing clearance of parasites and allergic reactions (Li et al., 2011), were reduced in LPS-stimulated leukocytes from male treated rats. However, no significant differences were found in female animals.

Our results suggest that the blockage of neonatal leptin surge have led to impairments both on Th1 and Th2 responses, although with a greater impact on Th1, since statistically significant differences were greater in Th1 cytokines, such as IL-2, IL-12p70 and IFN-γ, than in Th2 cytokines (IL-4 and IL-5). In this sense, previous data have pointed that the reduction of leptin concentrations (commonly seen in conditions of malnutrition, anorexia nervosa or genetic leptin deficiency) results in impaired Th1 response and reduced immunocompetence as well as increased susceptibility to infection (Procaccini et al., 2012).

Given that previous studies have shown the activation of JAK-2/STAT, MAPK and PI3K signaling pathways by leptin (Sánchez-Margalet et al., 2003), we could suggest a possible involvement of these intracellular pathways in the alterations of immune and inflammatory responses of peripubertal rats neonatally treated with a leptin antagonist.

In conclusion, these data indicate that the blockage of the neonatal leptin surge results in an impairment of immune function and in an altered cytokine release of spleen leukocytes peripubertal male and female rats. Therefore, the neonatal leptin surge seems to have an important physiological role in the establishment and maintenance of adequate immune response. Given the critical role of the immune system in maintaining health during adulthood and the aging process (De la Fuente and Miquel, 2009; Hunsche et al., 2016), it is reasonable to assume that any disruption/blockage of the neonatal leptin surge could represent a risk factor for the proper maintenance of the immune system function as well as overall health in later life.

Acknowledgements

This work was supported by grants from Ministry of Science and Innovation (BFU2011-30336), the Research Group of Madrid Complutense University (910379ENEROINN), Red Temática de Investigación Cooperativa en Envejecimiento y Fragilidad (RETICEF RD12/0043/0018) and FIS (PI15/01787) of Institute de Salud Carlos III – Fondo Europeo de Desarrollo Regional (ISCIII-FEDER).

C.H. is the recipient of a PhD fellowship from CNP-q-Brazil.

Conflict of interest

The authors declare no competing or financial interests.

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573 **FIGURE LEGENDS**

574

575 **Figure 1. Lymphocyte immune functions.** Natural killer cell activity (% lysis) in peripubertal female (A) and male
576 rats (C) treated neonatally with a leptin antagonist or vehicle (control). Chemotaxis index in peripubertal female (B) and
577 male rats (D) treated neonatally with a leptin antagonist or vehicle (control). ConA-stimulated proliferation in
578 peripubertal female (E) and male rats (F). LPS-stimulated proliferation in peripubertal female (G) and male rats (H)
579 treated neonatally with a leptin antagonist or vehicle (control). * $P<0.05$, *** $P<0.001$ with respect to the values of
580 control rats.

581

582 **Figure 2. IL-2 concentrations (pg/ml) in supernatants of spleen leukocytes cultures under concalavalin A (ConA)**
583 **and lipopolysaccharide (LPS)-stimulated conditions.** IL-2 concentrations (ConA) in peripubertal female (A) and
584 male rats (C) treated neonatally with a leptin antagonist or vehicle (control). IL-2 concentrations (LPS) in peripubertal
585 female (B) and male rats (D) treated neonatally with a leptin antagonist or vehicle (control). * $P<0.05$, ** $P<0.01$, ***
586 $P<0.001$ with respect to the values of control rats.

587

588 **Figure 3. IL-1 family concentrations (pg/ml) in supernatants of spleen leukocytes cultured under concalavalin A**
589 **(ConA) and lipopolysaccharide (LPS)-stimulated conditions.** IL-1α concentrations (ConA) in peripubertal female
590 (A) and male rats (C) treated neonatally with a leptin antagonist or vehicle (control). IL-1α concentrations (LPS) in
591 peripubertal female (B) and male rats (D) treated neonatally with a leptin antagonist or vehicle (control). IL-1β
592 concentrations (ConA) in peripubertal female (E) and male rats (G) treated neonatally with a leptin antagonist or vehicle
593 (control). IL-1β concentrations (LPS) in peripubertal female (F) and male rats (H) treated neonatally with a leptin
594 antagonist or vehicle (control). * $P<0.05$, ** $P<0.01$ with respect to the values of control rats.

595

596 **Figure 4. IL-6 concentrations (pg/ml) in supernatants of spleen leukocytes cultures under concalavalin A (ConA)**
597 **and lipopolysaccharide (LPS)-stimulated conditions.** IL-6 concentrations (ConA) in peripubertal female (A) and
598 male rats (C) treated neonatally with a leptin antagonist or vehicle (control). IL-6 concentrations (LPS) in peripubertal
599 female (B) and male rats (D) treated neonatally with a leptin antagonist or vehicle (control). * $P<0.05$, *** $P<0.001$
600 with respect to the values of control rats.

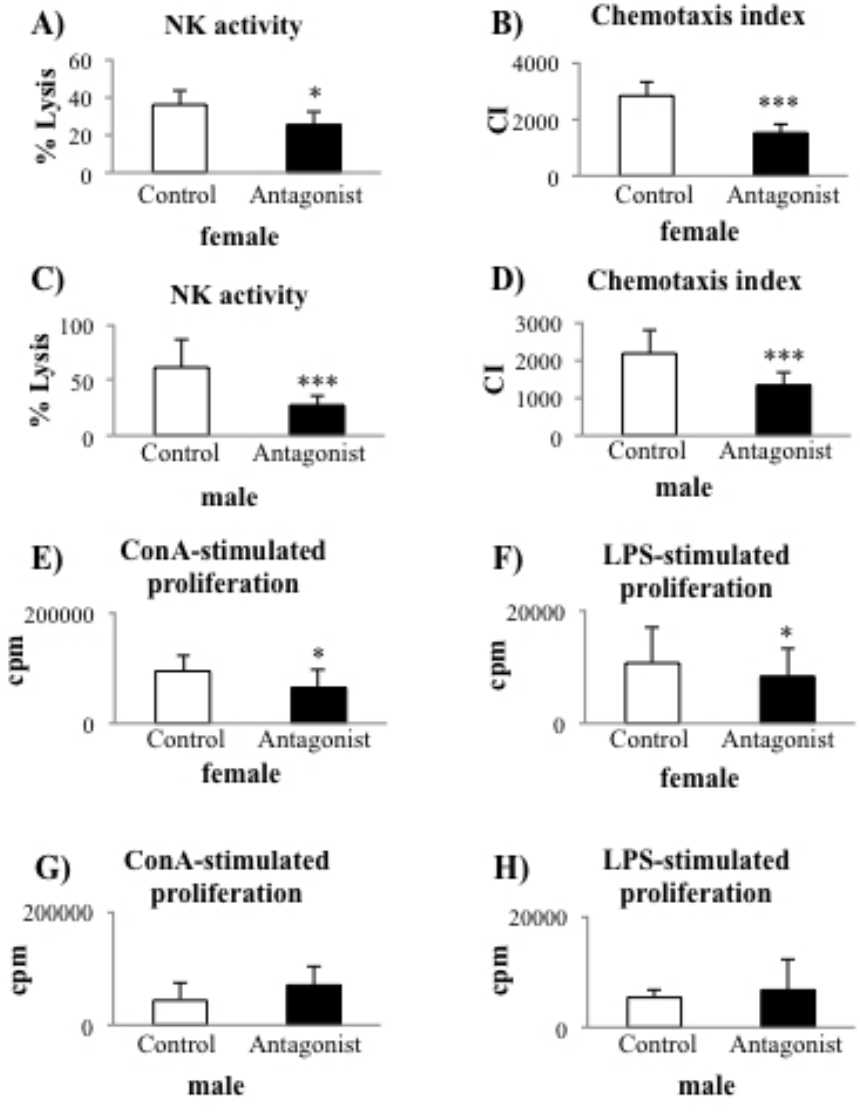
601

602 **Figure 5. IL-12p70 and IFN-γ concentrations (pg/ml) in supernatants of spleen leukocytes cultures under**
603 **concalavalin A (ConA) and lipopolysaccharide (LPS)-stimulated conditions.** IL-12p70 concentrations (ConA) in
604 peripubertal female (A) and male rats (C) treated neonatally with a leptin antagonist or vehicle (control). IL-12p70

concentrations (LPS) in peripubertal female (B) and male rats (D) treated neonatally with a leptin antagonist or vehicle (control). IFN-γ concentrations (ConA) in peripubertal female (E) and male rats (G) treated neonatally with a leptin antagonist or vehicle (control). IFN-γ concentrations (LPS) in peripubertal female (F) and male rats (H) treated neonatally with a leptin antagonist or vehicle (control). * $P<0.05$, ** $P<0.01$ with respect to the values of control rats.

Figure 6. GM-CSF concentrations (pg/ml) in supernatants of spleen leukocytes cultures under concalavalin A (ConA) and lipopolysaccharide (LPS)-stimulated conditions. GM-CSF concentrations (ConA) in peripubertal female (A) and male rats (C) treated neonatally with a leptin antagonist or vehicle (control). GM-CSF concentrations (LPS) in peripubertal female (B) and male rats (D) treated neonatally with a leptin antagonist or vehicle (control). t $0.05<P<0.1$, * $P<0.05$ with respect to the values of control rats.

Figure 7. IL-4 and IL-5 concentrations (pg/ml) in supernatants of spleen leukocytes cultures under concalavalin A (ConA) and lipopolysaccharide (LPS)-stimulated conditions. IL-4 concentrations (ConA) in peripubertal female (A) and male rats (C) treated neonatally with a leptin antagonist or vehicle (control). IL-4 concentrations (LPS) in peripubertal female (B) and male rats (D) treated neonatally with a leptin antagonist or vehicle (control). IL-5 concentrations (ConA) in peripubertal female (E) and male rats (G) treated neonatally with a leptin antagonist or vehicle (control). IL-5 (LPS) in peripubertal female (F) and male rats (H) treated neonatally with a leptin antagonist or vehicle (control). * $P<0.05$ with respect to the values of control rats.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NK activity
% Lysis
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40
20
0
*
Control
Antagonist
female
B)
Chemotaxis index
CI
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0
***
Control
Antagonist
female
C)
NK activity
% Lysis
100
50
0
***
Control
Antagonist
male
D)
Chemotaxis index
CI
3000
2000
1000
0
***
Control
Antagonist
male
E)
ConA-stimulated proliferation
cpm
200000
0
*
Control
Antagonist
female
F)
LPS-stimulated proliferation
cpm
20000
0
*
Control
Antagonist
female
G)
ConA-stimulated proliferation
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Control
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H)
LPS-stimulated proliferation
cpm
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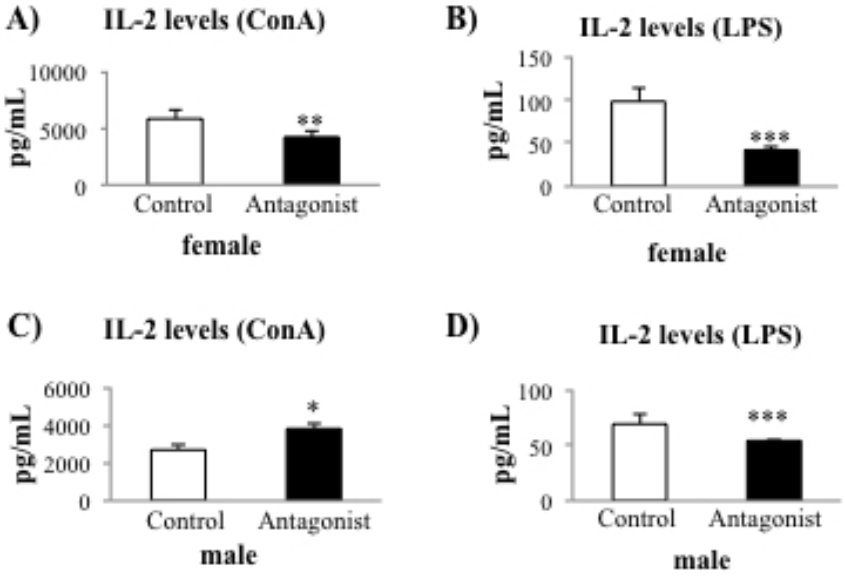
Antagonist
male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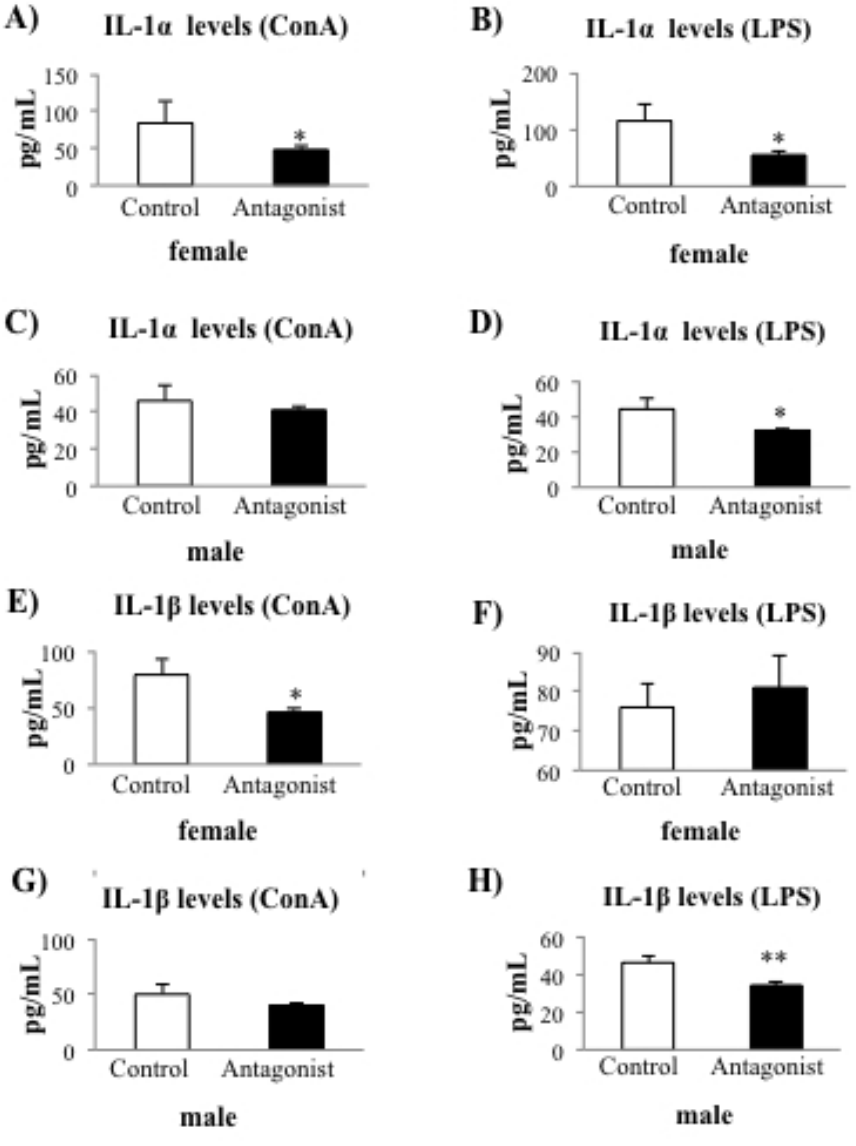
pg/mL
**
Control
Antagonist
female
B)
IL-2 levels (LPS)
150
100
50
0
pg/mL
***
Control
Antagonist
female
C)
IL-2 levels (ConA)
6000
4000
2000
0
pg/mL
*
Control
Antagonist
male
D)
IL-2 levels (LPS)
100
50
0
pg/mL
***
Control
Antagonist
male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pg/mL
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100
50
0
*
Control
Antagonist
female
B)
IL-1α levels (LPS)
pg/mL
200
100
0
*
Control
Antagonist
female
C)
IL-1α levels (ConA)
pg/mL
60
40
20
0
Control
Antagonist
male
D)
IL-1α levels (LPS)
pg/mL
60
40
20
0
*
Control
Antagonist
male
E)
IL-1β levels (ConA)
pg/mL
100
50
0
*
Control
Antagonist
female
F)
IL-1β levels (LPS)
pg/mL
90
80
70
60
Control
Antagonist
female
G)
IL-1β levels (ConA)
pg/mL
100
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0
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male
H)
IL-1β levels (LPS)
pg/mL
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0
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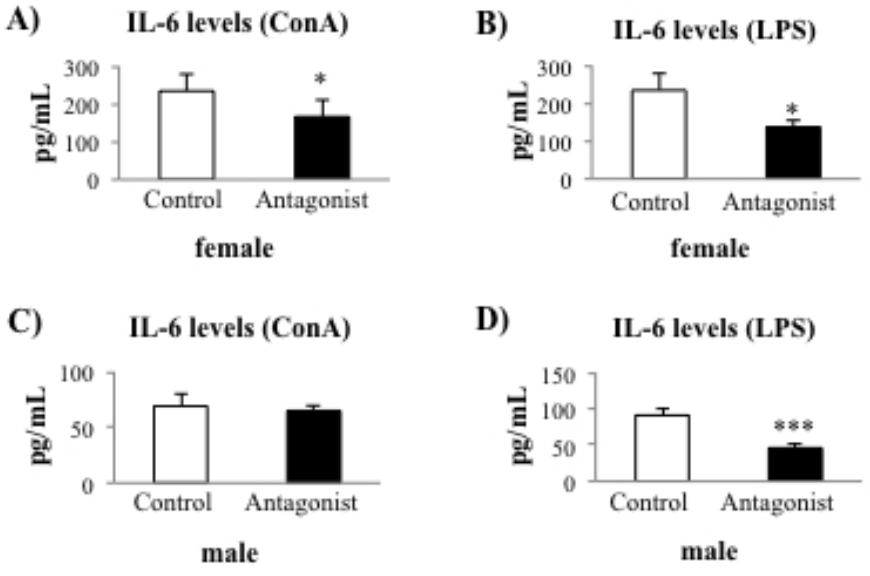
Control
Antagonist
male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pg/mL
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200
100
0
*
Control
Antagonist
female
B)
IL-6 levels (LPS)
pg/mL
300
200
100
0
*
Control
Antagonist
female
C)
IL-6 levels (ConA)
pg/mL
100
50
0
Control
Antagonist
male
D)
IL-6 levels (LPS)
pg/mL
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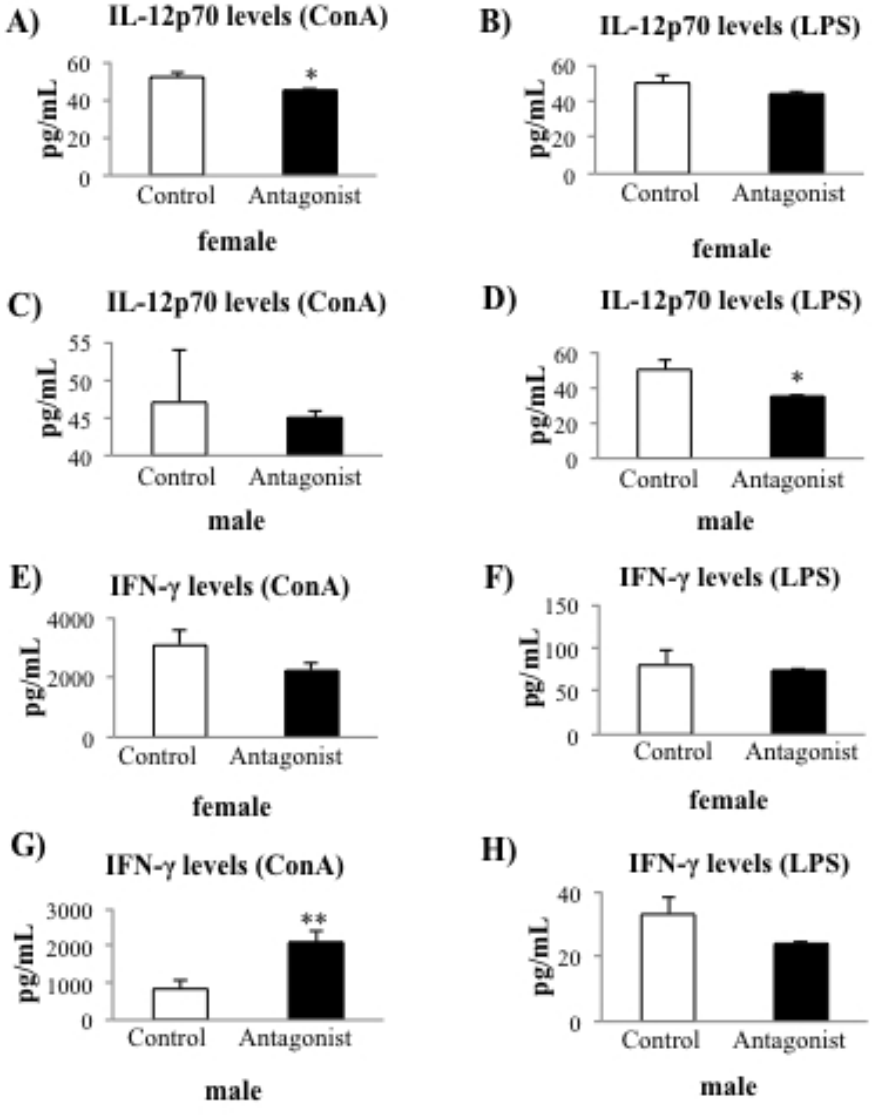
50
0
***
Control
Antagonist
male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female
B)
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pg/mL
60
40
20
0
Control
Antagonist
female
C)
IL-12p70 levels (ConA)
pg/mL
55
50
45
40
Control
Antagonist
male
D)
IL-12p70 levels (LPS)
pg/mL
60
40
20
0
*
Control
Antagonist
male
E)
IFN-γ levels (ConA)
pg/mL
4000
2000
0
Control
Antagonist
female
F)
IFN-γ levels (LPS)
pg/mL
150
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50
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Control
Antagonist
female
G)
IFN-γ levels (ConA)
pg/mL
3000
2000
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Antagonist
male
H)
IFN-γ levels (LPS)
pg/mL
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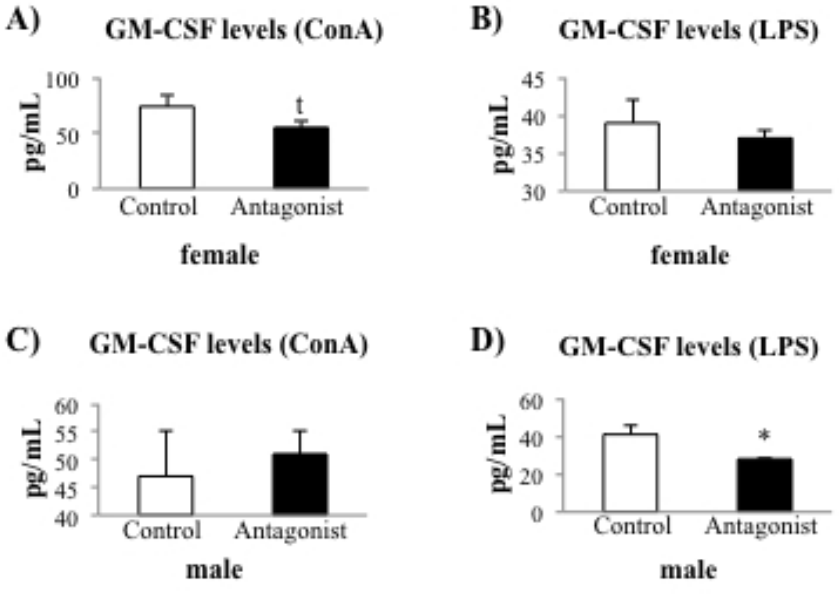
Control
Antagonist
male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0
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Control
Antagonist
female
B)
GM-CSF levels (LPS)
pg/mL
45
40
35
30
Control
Antagonist
female
C)
GM-CSF levels (ConA)
pg/mL
60
55
50
45
40
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male
D)
GM-CSF levels (LPS)
pg/mL
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40
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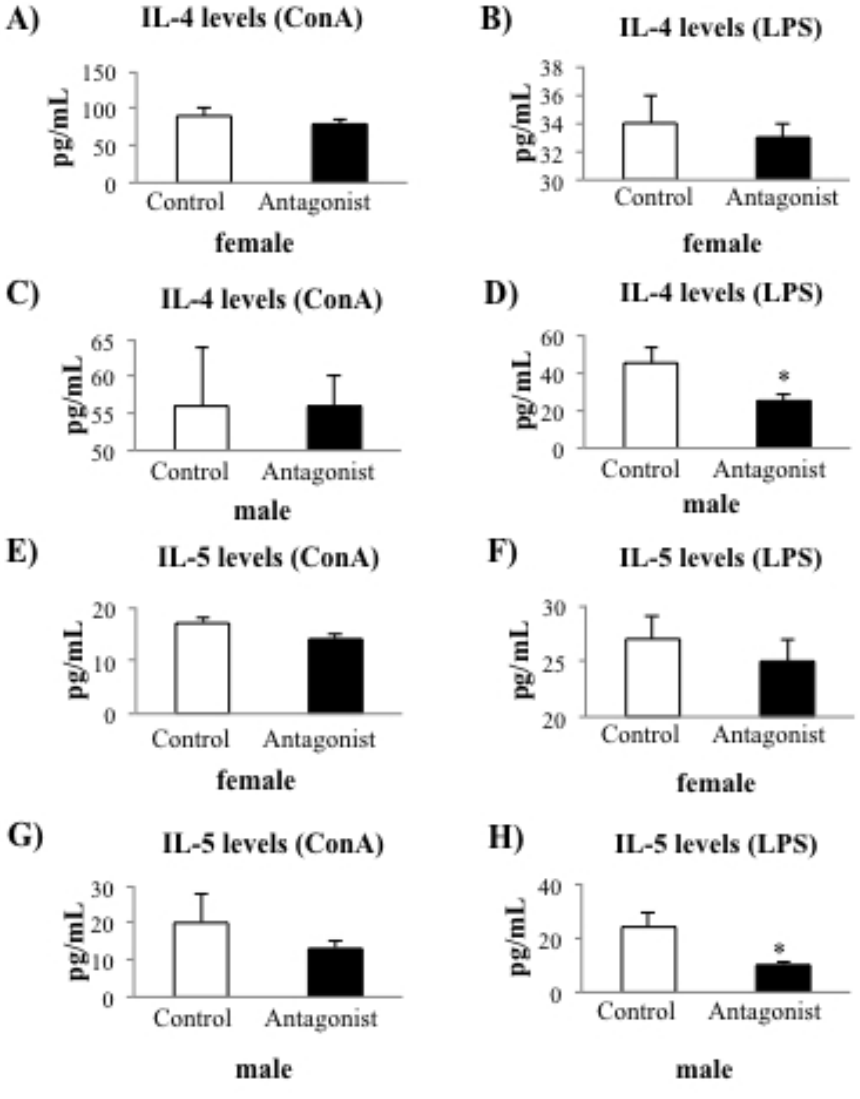
Antagonist
male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50
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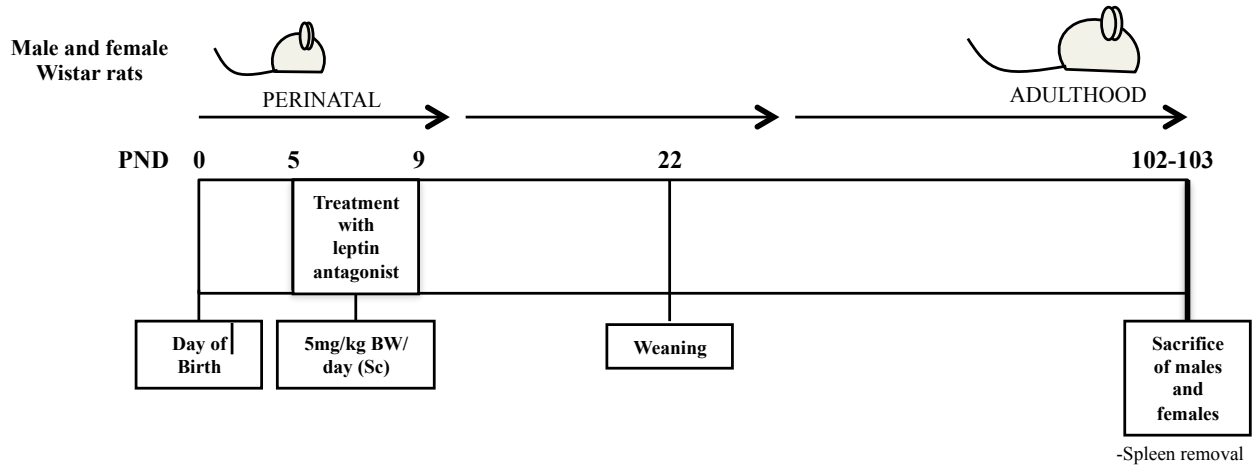
Antagonist
female
B)
IL-4 levels (LPS)
pg/mL
38
36
34
32
30
Control
Antagonist
female
C)
IL-4 levels (ConA)
pg/mL
65
60
55
50
Control
Antagonist
male
D)
IL-4 levels (LPS)
pg/mL
60
40
20
0
*
Control
Antagonist
male
E)
IL-5 levels (ConA)
pg/mL
20
10
0
Control
Antagonist
female
F)
IL-5 levels (LPS)
pg/mL
30
25
20
Control
Antagonist
female
G)
IL-5 levels (ConA)
pg/mL
30
20
10
0
Control
Antagonist
male
H)
IL-5 levels (LPS)
pg/mL
40
20
0
*
Control
Antagonist
male

3.2.3. Effects of the blockage of the neonatal leptin surge (PND5-9) on the immune function and redox state in spleen leukocytes of adult male and female rats.



Experimental design

From PND5 until PND9 rats were injected subcutaneously with pegylated super leptin antagonist (mutant D23L/L39A/D40A/F41A) (5 mg/kg bodyweight (bw) of rat). In order to consider the stress effect caused by marking the animals at this early age, each litter was treated with either vehicle (controls) or antagonist. The animals received one injection per day at 9:00 am. Control rats were injected with the same volume of distilled water as the vehicle (2.5 ml/kg). After each injection the animals were immediately returned to their mothers. Each experimental group consisted of 12 animals. To avoid/minimize possible litter effect, all experimental groups contained animals from at least three different litters. Male and female rats were sacrificed by rapid decapitation on PND102-103 after a 12 h fast. The spleen was rapidly and aseptically removed, freed of fat, minced with scissors, and gently pressed through a mesh screen to obtain the cell suspensions. The cell suspensions were centrifuged in a gradient of Ficcoll-Hypaque and used in the present sub-objective to evaluate immune function and redox state of spleen leukocytes in adult rats.

Main results

Spleen leukocyte function parameters

Adult male and female rats exposed to the leptin antagonist in the neonatal period showed significantly higher values of chemotaxis capacity of spleen leukocyte cells than those in controls. However, the anti-tumour NK activity showed lower values in treated male and female rats than in controls.

The lymphoproliferation in response to ConA was significantly lower in female and male treated rats than in controls. In addition, both treated adult males and females displayed lower values in lymphoproliferation in response to LPS with respect to controls.

Spleen leukocyte oxidative stress parameters

Reduced glutathione (GSH) and its related enzymes, including glutathione peroxidase (GPx) and glutathione reductase (GR) constitute the major intracellular redox buffer for cells against oxidant damage. The neonatal treatment with leptin antagonist resulted in a lower glutathione system antioxidant defence in adult rats. Thus, treated adult male and female rats showed lower GSH concentrations and GPx and GR activities.

With regards to the production of oxidants, adult male and female rats exhibited significantly higher concentrations of oxidized glutathione (GSSG) than their respective controls. In addition, the GSSG/GSH ratios, which are an indicator of oxidative stress, were significantly higher in adult male and female rats treated neonatally with the leptin antagonist than in controls. The activity of xanthine oxidase was significantly higher in treated adult male and female rats as compared with controls. In addition, the MDA concentration, which is an indicator of lipid oxidation and oxidative damage in cells, showed significantly higher values in treated adult female rats with respect to control female rats. Nevertheless, there were no significant differences in MDA concentrations between male treated rats and non-treated controls.

Partial conclusions

The results of this experiment indicate that the blockage of the neonatal leptin surge results in an impaired immune function and an altered redox state of spleen leukocytes in adult male and female rats.

Journal of Physiology and Biochemistry

LONG-TERM EFFECTS OF POSTNATAL LEPTIN DISRUPTION ON PARAMETERS OF IMMUNE FUNCTION AND OXIDATIVE STRESS IN SPLEEN OF RATS

--Manuscript Draft--

Manuscript Number:	JPBY-D-19-00219
Full Title:	LONG-TERM EFFECTS OF POSTNATAL LEPTIN DISRUPTION ON PARAMETERS OF IMMUNE FUNCTION AND OXIDATIVE STRESS IN SPLEEN OF RATS
Short Title:	Neonatal leptin surge, immune response and redox state
Article Type:	Original Article
Section/Category:	Endocrine Physiology - Mª Jesús Moreno
Keywords:	Neonatal leptin surge; Leptin antagonist; Immune functions; Oxidative stress; Adult male and female rats
Corresponding Author:	Monica De la Fuente, Ph.D.,M.D. Complutense University of Madrid Madrid, Madrid SPAIN
Corresponding Author Secondary Information:	
Corresponding Author's Institution:	Complutense University of Madrid
Corresponding Author's Secondary Institution:	
First Author:	Caroline Hunsche
First Author Secondary Information:	
Order of Authors:	Caroline Hunsche
	Oskarina Hernandez
	Virginia Mela
	M. Paz Viveros
	Monica De la Fuente
Order of Authors Secondary Information:	
Funding Information:	Ministerio de Ciencia e Innovación (BFU2011-30336) — Mrs Caroline Hunsche
	Research Group of Madrid Complutense University (910379ENEROINN) — Mrs Caroline Hunsche
	Instituto de Salud Carlos III (RETICEF RD12/0043/0018) — Mrs Caroline Hunsche
	Institute de Salud Carlos III (PI15/01787) — Mrs Caroline Hunsche
Abstract:	Most of studies have focused on the association between neonatal leptin surge and the development of nervous central circuits that regulate energy homeostasis. However, new evidence has emerged suggesting that the surge of leptin could also have an important role in the establishment and development of the immune system. We previously reported that leptin surge disruption affected several immune functions and oxidative-inflammatory parameters in peripubertal rats. In the present study, we further investigated, in adult male and female rats, whether the use of a leptin antagonist during the postnatal surge of leptin could affect relevant parameters of immune and redox state of spleen leukocytes. Male and female rats were neonatally treated with a specific leptin antagonist from postnatal day (PND) 5 to 9 and several parameters of immunity and oxidative stress state were measured at adult age. The results showed that adult male and female rats exposed to this leptin antagonist displayed significantly impaired chemotaxis capacity, anti-tumor NK activity and proliferation of lymphocytes

	in response to the mitogens concanavalin A and lipopolysaccharide. In addition, these animals showed higher oxidant parameters (xanthine oxidase activity and oxidized glutathione concentrations) and lower antioxidant defense (reduced glutathione concentrations and its related enzymes glutathione peroxidase and glutathione reductase activities) with higher lipid peroxidation (malondialdehyde) amounts than the corresponding controls. In conclusion, the disruption of the neonatal leptin surge leads to long-term impaired immune functions and increased oxidative stress state of spleen leukocytes of adult male and female rats.
Suggested Reviewers:	Radheshyam Maurya Professor, University of Hyderabad rmusl@uohyd.ernet.in Similar research interests
	Takehiko Maeda Professor, Niigata University tmaeda@wakayama-med.ac.jp Similar research interests
	Jun Ren Professor, University of Wyoming jren@uwyo.edu Similar research interests

Madrid, June 4th, 2019

Dear Editor-in-Chief,

We are pleased to submit the manuscript entitled "Long-term effects of postnatal leptin disruption on parameters of immune function and oxidative stress in spleen of rats", for its possible publication in Journal of Physiology and Biochemistry.

This is an original study focused on the effects that the exposure to a leptin antagonist during the neonatal period could have on immune function and redox state in spleen leukocytes of adult male and female rats.

This paper should be of interest to readers of Journal of Physiology and Biochemistry since it provides evidence that that the blockage of the neonatal leptin surge leads to long-term impaired immune functions and altered redox state in adult male and female rats. Therefore, the neonatal leptin surge seems to have an important physiological role in the establishment and maintenance of adequate immune response and redox state.

We state that the data presented in the manuscript have not been published elsewhere, in whole or in part, and that this work is not currently under review or accepted for publication in other journals. All authors have contributed to the final manuscript and all authors have read, approved and agreed to submit the manuscript to Journal of Physiology and Biochemistry.

Yours sincerely,

Mónica De la Fuente

Department of Genetic, Physiology and Microbiology. Faculty of Biology, Complutense University of Madrid
José Antonio Novais Street, 12. 28040. Madrid. Spain.
Telephone number: 0034 91 3944989
Fax number: 0034 91 3944935
Email address: mondelaf@bio.ucm.es

LONG-TERM EFFECTS OF POSTNATAL LEPTIN DISRUPTION ON PARAMETERS OF IMMUNE FUNCTION AND OXIDATIVE STRESS IN SPLEEN OF RATS

Caroline Hunsche[1,2], Oskarina Hernandez[1], Virginia Mela[1], M. Paz Viveros[1], Mónica De la Fuente[1,2]

[1]Department of Genetic, Physiology and Microbiology. Faculty of Biology, Complutense University of Madrid. José Antonio Novais Street, 12. 28040. Madrid. Spain.

[2]Research Institute of Hospital 12 de Octubre (i+12).

***Corresponding author:**

Mónica De la Fuente

Department of Genetics, Physiology and Microbiology. Faculty of Biology. Complutense University of Madrid

José Antonio Novais Street, 2. 28040. Madrid. Spain.

Telephone number: 0034 91 3944989

Fax number: 0034 91 3944935

Email address: mondelaf@bio.ucm.es

Abstract

Most of studies have focused on the association between neonatal leptin surge and the development of nervous central circuits that regulate energy homeostasis. However, new evidence has emerged suggesting that the surge of leptin could also have an important role in the establishment and development of the immune system. We previously reported that leptin surge disruption affected several immune functions and oxidative-inflammatory parameters in peripubertal rats. In the present study, we further investigated, in adult male and female rats, whether the use of a leptin antagonist during the postnatal surge of leptin could affect relevant parameters of immune and redox state of spleen leukocytes. Male and female rats were neonatally treated with a specific leptin antagonist from postnatal day (PND) 5 to 9 and several parameters of immunity and oxidative stress state were measured at adult age. The results showed that adult male and female rats exposed to this leptin antagonist displayed significantly impaired chemotaxis capacity, anti-tumor NK activity and proliferation of lymphocytes in response to the mitogens concanavalin A and lipopolysaccharide. In addition, these animals showed higher oxidant parameters (xanthine oxidase activity and oxidized glutathione concentrations) and lower antioxidant defense (reduced glutathione concentrations and its related enzymes glutathione peroxidase and glutathione reductase activities) with higher lipid peroxidation (malondialdehyde) amounts than the corresponding controls. In conclusion, the disruption of the neonatal leptin surge leads to long-term impaired immune functions and increased oxidative stress state of spleen leukocytes of adult male and female rats.

Keywords: Neonatal leptin surge; Leptin antagonist; Immune functions; Oxidative stress; Adult male and female rats

- Neonatal blockage of leptin surge impairs immune response of adult rats.
- Neonatal blockage of leptin surge increases oxidative stress of adult rats.

1. Introduction

Leptin is a pleiotropic hormone with not only an important role in the central regulation of energy homeostasis, but also involved in other peripheral processes, such as the modulation of immune system functions [19]. Leptin is predominantly secreted by adipocytes in adipose tissue but at low amounts also by skeletal muscle, stomach and placenta [4,28,29,33]. During the gestational period, the expression of leptin and its receptor significantly increases in placenta and fetal tissues of humans and rodents [28,29]. Thus, these findings suggest that leptin has an important role in the development of fetus. In addition, there is a transient 5-to 10-fold increase of plasma leptin concentrations in mice and rats during the early postnatal period, which has been termed as leptin surge. Both male and female rodents experience elevated levels of leptin during the first two weeks after birth, followed by a decline in its levels at weaning [6,12]. Particularly, this leptin surge has shown to be important for the establishment of hypothalamic circuits that regulate energy homeostasis and reproduction [4,23]. Increasing evidence suggests that early life is a critical developmental period and any stress generated in this period could result in long-term negative effects in later life [21]. Indeed, the disruption of neonatal levels of leptin has been associated with long-term impairments in energy homeostasis and reproduction [4,23].

Increasing evidence suggests that leptin is able to modulate a variety of innate and adaptive immune responses, including the migration and proliferation of immune cells and the antitumor activity of natural killer (NK) cells [20,22,26]. The expression of leptin receptors by several immune cells, such as macrophages, monocytes, neutrophils, NK cells, T and B cells confirms the important role that leptin plays in the regulation of immune responses [20,31]. In addition, leptin is able to promote the production of reactive oxygen species (ROS) [9,34]. ROS generation are involved in various important physiological processes of the body, including the immune response. However, under certain conditions of metabolic stress, an imbalance between ROS and antioxidant defenses in favor of ROS production may lead to oxidative stress [18]. The enhancement of oxidative stress has potentially deleterious effects, such as immune system dysfunction. In addition, increased oxidative stress state throughout life is believed to contribute to the aging process [18]. In fact, increased oxidative stress state, dysfunctional immune response as well as abnormal leptin levels were found to be common features of aging and many pathological conditions (such as obesity, insulin resistance and cardiovascular diseases) [6,16,32].

Most of studies have focused on the association between neonatal leptin surge and development of central nervous system [23]. However, emerging evidence suggests that the surge of leptin could also have an important role in the establishment and development of the immune system. In this regard, a previous study demonstrated that the blockage of leptin levels during the early neonatal period resulted in dysfunctional maturation of key immune organs, such as thymus and spleen [3]. In addition, we also showed that the exposure to a leptin antagonist during the postnatal

period resulted in impaired immune function in spleen [unpublished data] as well as in increased inflammatory and oxidative state in spleen, hypothalamus and adipose tissue during the peripubertal/adolescence period of rats [24]. However, it remains unclear whether the blockage of the leptin surge has long-term effects on immune functions and oxidative stress state during adulthood. Therefore, in the present study, we further investigated if the use of a leptin antagonist during the period corresponding to the physiological surge of leptin (PND5-9) could affect relevant parameters of immune and redox state of spleen leukocytes in adult male and female rats.

2. Materials and methods

2.1. Animals

Experimental subjects were the offspring of Wistar rats purchased from Harlan Interfauna Ibérica SA (Barcelona, Spain). The parental generation was mated (one male x two females) in our animal facilities approximately two weeks after their arrival. After 10 days, female animals were isolated and the day of delivery strictly controlled. On the day of birth (postnatal day 0, PND0), litters were sex-balanced and culled to eight pups per dam (four males and four females). No cross-fostering was employed. Thus, only litters with at least four pups of each sex at birth were used. The animals were housed in plastic Makrolon® III cages and maintained at a constant temperature (22 ± 1°C) and humidity (50 ± 2%) in a reversed 12-h light-dark cycle (red light on at 8:00 am and white light on at 8:00 pm). All rats were given free access to rat chow (commercial diet for rodents, A03, Safe, Augy, France) and water.

These studies were approved by the local ethics committee and complied with Royal Decree 53/2005 (BOE n° 252) pertaining to the protection of experimental animals and with the European Communities Council Directive (86/609/EEC).

2.2. Leptin antagonist treatment

From PND5 until PND9 rats were injected subcutaneously with pegylated super leptin antagonist (mutant D23L/L39A/D40A/F41A) (5 mg/kg bodyweight (bw) of rat) a gift of Protein Laboratories (Rehovot, Israel), which was prepared as previously described [17]. In order to consider the stress effect caused by marking the animals at this early age, we treated each litter with either vehicle (controls) or antagonist. The animals received one injection per day at 9:00 am. Control rats were injected with the same volume of distilled water as the vehicle (2.5 ml/kg). After each injection the animals were immediately returned to their mothers. Each experimental group consisted of 12 animals. To avoid/minimize possible litter effect all experimental groups contained animals from at least three different litters.

2.3 Collection of spleen and leukocyte suspensions

Male and female rats were sacrificed by rapid decapitation on PND102-103 after a 12 h fast. The spleen was rapidly and aseptically removed. The spleen was then freed of fat and minced with scissors, and gently pressed through a mesh screen (Sigma-Aldrich, Madrid, Spain) to obtain the cell suspensions. The cell suspensions were centrifuged in a gradient of Ficoll-Hypaque (Sigma-Aldrich) with a density of 1.070 g/ml. Cells from the interface were collected and suspended in Roswell Park Memorial Institute (RPMI) 1640 medium enriched with L-glutamine (PAA, Pasching, Austria) and supplemented with 10% heat-inactivated (56 °C, 30 min) fetal calf serum (PAA) and gentamicin (100 µg/ml, PAA). After a wash step, leukocytes were counted in a Neubauer chamber (Blau Brand, Wertheim, Germany) and their number adjusted to 10^6 cells/ml. Cell viability was routinely measured before each experiment by the trypan-blue exclusion test, and was higher than 98% in all experiments. All incubations were performed at 37 °C in a humidified atmosphere of 5% CO_2.

2.4. Immune functions

2.4.1. NK cell activity assay

An enzymatic colorimetric assay was carried out to measure the cytolysis of tumor cells (Cytotox 96 TM Promega, Boehringer Ingelheim, Germany) based on the determination of lactate dehydrogenase enzyme (LDH), as previously described [10]. Aliquots of 100 µl of spleen leukocytes, used as effector cells, were seeded in 96-well U-bottom culture plates (Numc, Roskilde, Denmark) adjusted to 10^6 leukocytes/ml in RPMI 1640 medium without phenol red. Murine lymphoma YAC-1 cells, used as target cells, were added adjusted to 10^5 cells/ml. Thus, the effector/target ratio was 10:1. The plates were centrifuged at 250 g for 4 min to facilitate cell contacts. After 4 hours of incubation, lactate dehydrogenase enzymatic activity was measured in 50 µl/well of the supernatants by addition of the enzyme substrate and absorbance recording spectrophotometrically at 490 nm. Three kinds of control measurements were performed: a target spontaneous release, a target maximum release, and an effector spontaneous release. The results were expressed as percentages of lysis of target cells. To determine this percentage the following equation was used: %lysis = ((E-ES-TS)/M-ES-TS)) x 100, where E is the mean of absorbance in the presence of effector cells; ES, the mean of absorbance of effector cells incubated alone; TS, the mean of absorbance in target cells incubated with medium alone; and M is the mean of maximum absorbance after incubating target cells with lysis solution.

2.4.2. Chemotaxis assay

Chemotaxis of spleen leukocytes was evaluated according to a slight modification of Boyden´s method [10] consisting basically of the use of chambers with two compartments separated by a filter with a pore diameter of 3 µm (Millipore, Bedford, MA, USA). Aliquots of 300 µl of the spleen leukocyte suspensions, adjusted to $5X10^5$ cells/ml in Hank´s solution, were deposited in the upper compartment, and aliquots of 400 µl of the chemoattractant, formyl-Met-Leu-Phe (10^{-8} M) (Sigma-Aldrich), were put into the lower compartment. The chambers were incubated for 3h and then the filters were fixed and stained. The chemotaxis index (CI) was determined by counting, in an optical microscope (100X), the total number of lymphocytes on one third of the lower face of the filters.

2.4.3. Lymphoproliferation assay

The proliferation of lymphocytes in response to the mitogens concanavalin A (ConA) and lipopolysaccharide (LPS) was measured following a method previously described [10]. Aliquots (200 µl) of spleen leukocytes (10^6 cells/ml complete medium) were seeded in 96 well flat-bottomed plates (Numc, Roskilde, Denmark), and 20 µl of ConA (1µg/ml; Sigma-Aldrich), a T-cell mitogen or 20 µl of lipopolysaccharide (*Escherichia coli*, 055:B5 1µg/ml; Sigma-Aldrich), a B-cell mitogen, were added per well. After 48h of incubation at 37°C in an atmosphere of 5% CO2, 0.5 µCi ^{3}H-thymidine (Du Pont, Boston, MA, USA) were added to each well. The cells were harvested in a semiautomatic microharvester 24h later. Thymidine uptake was measured using a beta counter (LKB, Uppsala, Sweden) and the results were expressed as ^{3}H-thymidine uptake (cpm).

2.5. Oxidative stress parameters

2.5.1. Glutathione peroxidase activity

The glutathione peroxidase (GPx) activity was determined according to method previously described [1]. Aliquots of 1 ml of spleen leukocytes adjusted to 10^6 cells/ml of Hank´s solution were centrifuged at 1200 g for 10 min at 4°C. The pellets of cells were resuspended in phosphate buffer (50mM, pH 7.4, Sigma-Aldrich). Then, the samples were sonicated and centrifuged at 3200 g for 20 minutes at 4°C. The total activity was determined using cumene hydroperoxide (cumene-OOH, Sigma-Aldrich), which carried out the oxidation of the glutathione regenerated by the addition of β-nicotinamide adenine di-nucleotide phosphate, in its reduced form (β-NADPH, Sigma-Aldrich), in the presence of glutathione reductase (Sigma-Aldrich). The reaction was followed spectrophotometrically by the decrease

of the absorbance at 340 nm. The results were expressed as international milliunits (mU) of enzymatic activity per 10^6 cells.

2.5.2. Glutathione reductase activity

The glutathione reductase (GR) activity was measured by a method previously described [1]. Aliquots of 1 ml of spleen leukocytes adjusted to 10^6 cells/ml of Hank´s solution were centrifuged at 1200 g for 10 min at 4°C. The pellets of cells were resuspended in phosphate buffer (50mM, pH 7.5, Sigma-Aldrich) containing ethylenediaminetetraacetic acid (EDTA) (6.3 mM, Sigma-Aldrich). Then, the samples were sonicated and centrifuged at 3200 g for 20 minutes at 4°C. The total activity was measured through the oxidation of NADPH spectrophotometrically at 340 nm. The results were expressed as international milliunits (mU) of enzymatic activity per 10^6 cells.

2.5.3. Glutathione concentrations

Both reduced (GSH) and oxidized (GSSG) concentrations of glutathione were measured using a fluorometric method. This method is based on the reaction of a fluorescence probe, o-phthaldialdehyde (OPT; Sigma-Aldrich), with GSH at pH 8 and with GSSG at pH 12, which generates a fluorescence derivative. Aliquots of 1 ml of spleen leukocytes adjusted to 10^6 cells/ml of Hank´s solution were centrifuged at 1200 g for 10 min at 4°C. The pellets of cells were resuspended in phosphate buffer containing EDTA (0.1 M, pH 8, Sigma-Aldrich). Then, samples were sonicated and proteins were precipitated by adding 5 µl of perchloric acid ($HClO_4$) (60%; Sigma-Aldrich). Spleen leukocytes samples were centrifuged at 9500 g for 10 min at 4°C and supernatants were maintained in ice for measurement of GSH and GSSG concentrations. For GSH contents determination, 10 µl of the supernatant, 190 µl of phosphate-EDTA buffer and 20 µl of OPT solution (1 mg/ml in methanol) were added to a 96-well black plate (Nunc, Roskilde, Denmark) and incubated at room temperature for 15 minutes. Fluorescence was determined in a plate reader (Fluostar Optima, BMG Labtech, Spain) using excitation at 350 nm and emission detection at 420 nm. For the determination of GSSG contents, 8 µl of N-ethylmaleimide (NEM, 0,04M, Sigma-Aldrich) was added to each well and incubated at room temperature for 30 minutes. Then, 182 µl of sodium hydroxide (NaOH) (0.1 N; Panreac Quimica SA, Barcelona, Spain) with 20 µl of OPT solution were added to a 96-well black plate. After incubation (room temperature, 15 min), fluorescence was measured as previously described for GSH determination. The results were expressed as nmol/10^6 cells. The GSSG/GSH ratio was then calculated for each sample.

2.5.4. Lipid peroxidation (MDA) levels

The estimation of malondialdehyde (MDA) in cells was evaluated using the commercial kit "MDA Assay Kit" (Biovision, Mountain View, CA, USA), which measures the reaction of MDA with thiobarbituric acid (TBA) and the MDA-TBA adduct formation. Aliquots of 1 ml of spleen leukocytes adjusted to 10^6 cells/ml of Hank´s solution were centrifuged at 1200 g for 10 min at 4°C. The pellets of cells were re-suspended in 300 µl of MDA lysis buffer with 3 µl BHT. Then, the cell suspensions were sonicated and centrifuged at 13000 g for 10 minutes at 4°C to remove insoluble material. The supernatant (200 µl) from each sample was added to 600 µl of TBA reagent. After the incubation at 95°C for 60 minutes, samples were cooled to room temperature in an ice bath for 10 minutes and then 200 µl of supernatant was dispensed into a 96-well plate. The MDA-TBA adduct was measured using a spectrophotometer at 532 nm of absorbance. The results were expressed as nmol/10^6 cells.

2.5.5. Xanthine oxidase activity

Xanthine oxidase (XO) activity was measured by fluorescence using a commercial kit "Amplex Red Xanthine/Xanthine Oxidase Assay Kit" (Molecular Probes, Paisley, UK). The hydrogen peroxide (H_2O_2) produced by XO reacts with the horseradish peroxidase (HRP) present in the reaction mixture and generates a fluorescent oxidation compound resorufin whose fluorescence is measured in a plate reader (Fluostar Optima, BMG Labtech, Spain). Spleen leukocyte suspension (50 µl) adjusted to 10^6 cells/ml in Hank´s medium was incubated with 50 µl working solution of Amplex Red reagent (100 µM) containing HRP (0.4 U/ml) and xanthine (200 µM). After 30 minutes of incubation at 37°C, measurements of fluorescence was performed in a plate reader using excitation at 530 nm and emission detection at 595 nm. XO supplied in the kit was used as the standard, and XO activity was measured by comparing the fluorescence of samples with that of standards. The results were expressed in international milliunits (mU) of enzymatic activity per 10^6 cells.

2.6. Statistical analysis

SPSS 21.0 (SPSS, Inc., Chicago, USA) was used for the statistical analysis of the results. The data were expressed as mean ± standard deviation (SD). Each value is the mean of the data from an assay performed in duplicate or triplicate. Normality of the samples was checked by the Kolmogorov-Smirnov test and homogeneity of variances with the Levene test. The data were statistically evaluated by the Student´s *t*-test for independent samples. $P<0.05$ was considered as statistically significant.

3. Results

3.1. Immune functions

3.1.1. Lymphocyte innate immune functions

The innate immune functions of spleen leukocytes are shown in Figure 1. Adult male and female rats exposed to the leptin antagonist in the neonatal period showed significantly higher values of chemotaxis capacity of spleen leukocyte cells than those in controls ($P<0.05$. Figure 1A). The activity of NK cells showed lower values in treated male and female rats than in controls ($P<0.05$. Figure 1B).

3.1.2. Lymphocyte adaptive immune functions

The lymphoproliferation in response to T-cell mitogen (ConA) and B-cell mitogen (LPS) are shown in Figure 2. The lymphoproliferation in response to ConA was significantly lower in female and male treated rats than in controls ($P<0.01$ and $P<0.0001$, respectively. Figure 2A). In addition, both adult male and female showed lower levels in the lymphoproliferation in response to LPS in comparison with controls ($P<0.01$ and $P<0.001$, respectively. Figure 2B).

3.2. Oxidative stress parameters

3.2.1. Antioxidative parameters

Reduced glutathione (GSH) and its related enzymes, including glutathione peroxidase (GPx) and glutathione reductase (GR), which are shown in Figure 3, constitute the major intracellular redox buffer for cells against oxidant damage. The neonatal treatment with leptin antagonist resulted in lower glutathione system antioxidant defense in adult rats. Thus, adult male and female rats showed lower values of reduced glutathione concentration ($P<0.05$. Figure 3A) and of GPx ($P<0.05$. Figure 3B) and GR activities ($P<0.01$ and $P<0.05$, respectively. Figure 3C).

3.2.2. Oxidative parameters

Figure 4 shows oxidative parameters of spleen leukocytes. Adult male and female rats exhibited significantly higher concentration of oxidized glutathione (GSSG) than their respective controls ($P<0.05$ and $P<0.001$, respectively.

Figure 4A). In addition, the ratio between GSSG and GSH, which is an indicator of oxidative stress, was significantly higher in adult male and female rats treated neonatally with the leptin antagonist than in controls ($P<0.05$. Figure 4B).

The activity of xanthine oxidase (Figure 4C), which is associated with the production of free radicals, was significantly higher in adult male and female rats as compared with controls ($P<0.05$ and $P<0.01$, respectively).

The estimation of MDA levels (Figure 4D), which is an indicator of lipid oxidation and oxidative damage in cells, showed significantly higher values in adult female rats with respect to control female rats ($P<0.05$). Nevertheless, there were no significant differences in MDA levels between male treated rats and non-treated controls (Figure 4D).

4. Discussion

Although we previously demonstrated that the neonatal disruption of the leptin surge resulted in impairments of immune functions [unpublished data] and oxidative stress [24] during the peripubertal/adolescence period of rats. It seemed relevant to investigate whether this disruption could produce these effects at adult age (i.e., when the immune system has reached its complete development) [30]. The results of the present study found that the exposure to a leptin antagonist from PND 5 to 9, which is coincident with the neonatal leptin surge, resulted in long-term impaired immune response and redox state of spleen leukocytes of adult male and female rats. The immune functions and redox state were evaluated in the spleen, since this secondary lymphoid organ is essential for effective innate and adaptive immune responses in both humans and rodents [27].

With respect to the functions of the innate immunity studied, both the chemotaxis and anti-tumor NK activities, were significantly impaired in spleen leukocytes of adult male and female rats treated with a leptin antagonist. However, the neonatal leptin antagonist treatment resulted in chemotactic response differences between peripubertal/adolescent and adult rats. Thus, adult treated rats showed higher chemotactic response after peptide stimulation, whereas, in peripubertal/adolescent treated rats, this response was lower in comparison with their respective controls [unpublished data]. The higher chemotactic response in adults treated rats could be interpreted as a possible compensatory mechanism to compensate the lower response found in other immune cell functions, such as the NK cell activity [14]. In fact, the blockage of leptin in the neonatal period affected the NK activity so highly that not only did this function appear diminished at peripubertal age [unpublished data], but also at adult age. Previous studies demonstrated that leptin was able to modulate immune innate response, such as chemotaxis and NK cell activities. Therefore, leptin at low concentrations exhibited a chemoattractant capacity [14] as well as to stimulating the expression of chemokines in immune cells [18]. In addition, leptin seems to modulate the NK cell activity via the activator of transcription 3 (STAT-3) and by the up-regulation of IL-2 and perforin genes [13]. The lack of leptin

receptor expression in mice also results in impaired NK cell activity, which highlights the important role of leptin in regulating immune responses [12,13].

The neonatal administration of the leptin antagonist also resulted in long-term impaired adaptive immune response in adult rats. In agreement with our previous data found in peripubertal rats [unpublished data], the proliferation of spleen lymphocytes in response to T- and B-cell mitogens, such as ConA and LPS, respectively, continued to be lower in adult male and female treated rats. Increasing evidence indicates that leptin is able to modulate immune functions of lymphocytes. For instance, leptin receptor deficient mice displayed impaired lymphopoiesis and a low number of lymphocytes in peripheral blood, which indicates that leptin is important for the development of lymphocytes [7]. In addition, an *in vitro* study demonstrated that leptin increased the proliferation of lymphocytes in the presence of mitogens [22].

In the present study, we found long-term increased oxidative stress state in spleen leukocytes of adult treated rats. Thus, adult male and female rats neonatally treated with a leptin antagonist exhibited higher values of oxidant production, such as xanthine oxidase (XO) activity and GSSG contents, as well as lower concentrations of the antioxidant GSH and activities of its related antioxidant enzymes (GPx and GR) than those in the corresponding controls. Moreover, the GSSG/GSH ratios were higher, which show an oxidative stress [2] and consequently the amount of MDA, a lipid peroxidation marker, was also higher than in controls. These findings are partially in concordance with those found in a recently published study, which also evaluated a variety of oxidative stress parameters in the spleen leukocytes of peripubertal rats. Thus, in contrast to the results in adult rats, in which a lower activity of GR (an antioxidant enzyme) was observed, this activity was higher in treated peripubertal rats. The higher values of GR activity in treated peripubertal rats could be potentially explained as a compensatory mechanism in an attempt to restore redox homeostasis in animals treated with leptin antagonist. Similarly, the aging process, which has been linked to increased oxidative stress state, also described higher production of antioxidant enzymes as a compensatory mechanism [28]. Some studies have pointed to the possible involvement of leptin in modulating the oxidative stress response. Thus, in malnourished children with low amounts of leptin, enhanced lipid peroxidation as well as decreased antioxidant defense such as superoxide dismutase and catalase activities and GSH concentrations were observed [8]. Moreover, another study found that enhanced levels of leptin (in obesity and cardiovascular diseases) were also associated with increased production of ROS. It was demonstrated that leptin seems to increase ROS generation mainly by the activation of NADPH oxidase [13,25]. Therefore, it appears that abnormal circulating levels of leptin, either diminished or enhanced, could contribute to the imbalance of redox homeostasis. It is curious that the decrease of leptin levels in neonatal period allowed the maintenance of an oxidative stress in spleen at adult age.

In conclusion, the disruption of the neonatal leptin surge resulted not only in impaired immune functions and increased oxidative stress state in spleen leukocytes at peripubertal/adolescent age, as previously demonstrated

[unpublished data, 29], but also at adult age. Thus, these results indicate that the disruption of the neonatal leptin surge has long-term effects in immune function and oxidative stress parameters. Moreover, given that impaired immune system functions and increased oxidative stress generated at adult age have been associated with premature aging and a shorter life span [11], it is possible that the disruption of neonatal leptin levels could also have detrimental effects in the process of aging.

Acknowledgments

Caroline Hunsche is the recipient of a PhD fellowship from CNP-q-Brazil.

Funding

This work was supported by grants from Ministry of Science and Innovation (BFU2011-30336), the Research Group of Madrid Complutense University (910379ENEROINN), Red Temática de Investigación Cooperativa en Envejecimiento y Fragilidad (RETICEF RD12/0043/0018) and FIS (PI15/01787) of Institute de Salud Carlos III – Fondo Europeo de Desarrollo Regional (ISCIII-FEDER).

Compliance with Ethical Standards

The experiments were conducted in accordance with the guidelines and protocols of Royal Decree 53/2013 regarding the care and use of laboratory animals for experimental procedures, and were approved by the Committee for Animal Experimentation of the Complutense University of Madrid.

Conflict of interest

The authors declare no competing or financial interests.

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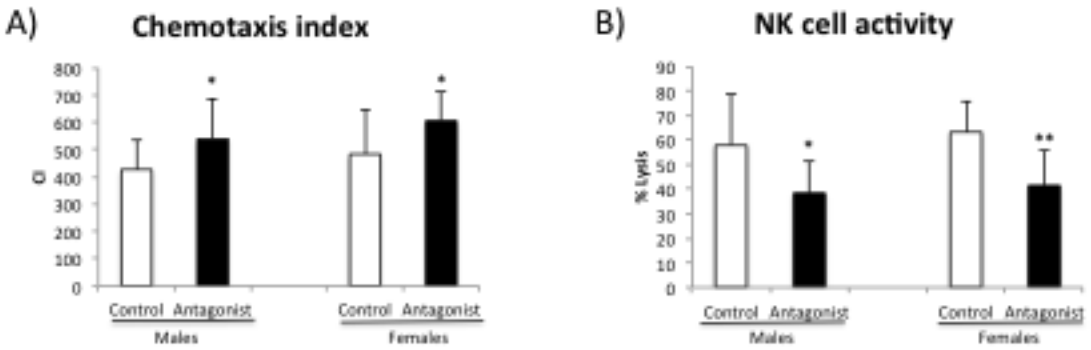
FIGURE LEGENDS

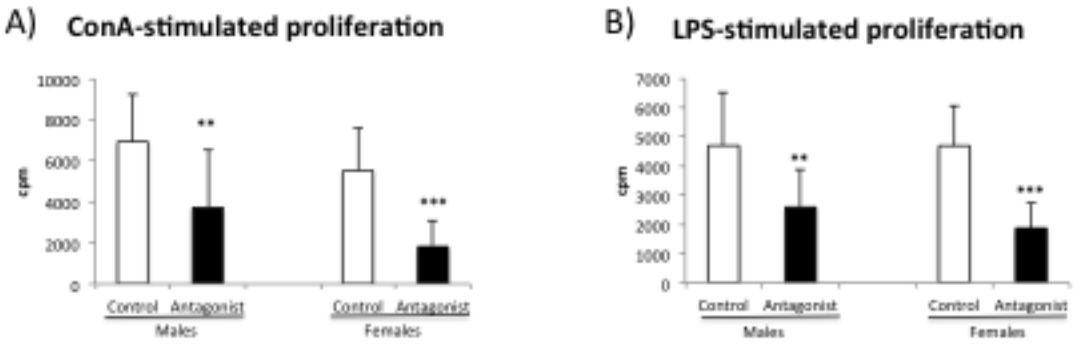
Figure 1. Lymphocyte innate functions. Chemotaxis index in adult male and female rats (A) treated neonatal with a leptin antagonist or vehicle (control). Natural killer cell activity (% lysis) in adult male and female rats (B) treated neonatal with a leptin antagonist or vehicle (control). * $P<0.05$, ** $P<0.01$ with respect to the values of control rats.

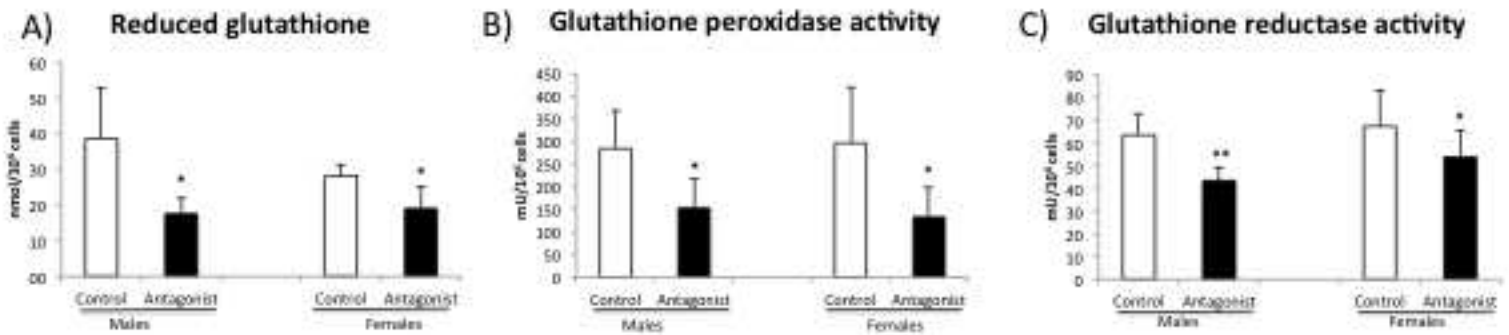
Figure 2. Lymphocyte adaptive immune functions. ConA-stimulated proliferation in adult male and female rats (A) treated neonatal with a leptin antagonist or vehicle (control). LPS-stimulated proliferation in adult male and female rats (B) treated neonatal with a leptin antagonist or vehicle (control). * $P<0.01$, ***$P<0.001$ with respect to the values of control rats.

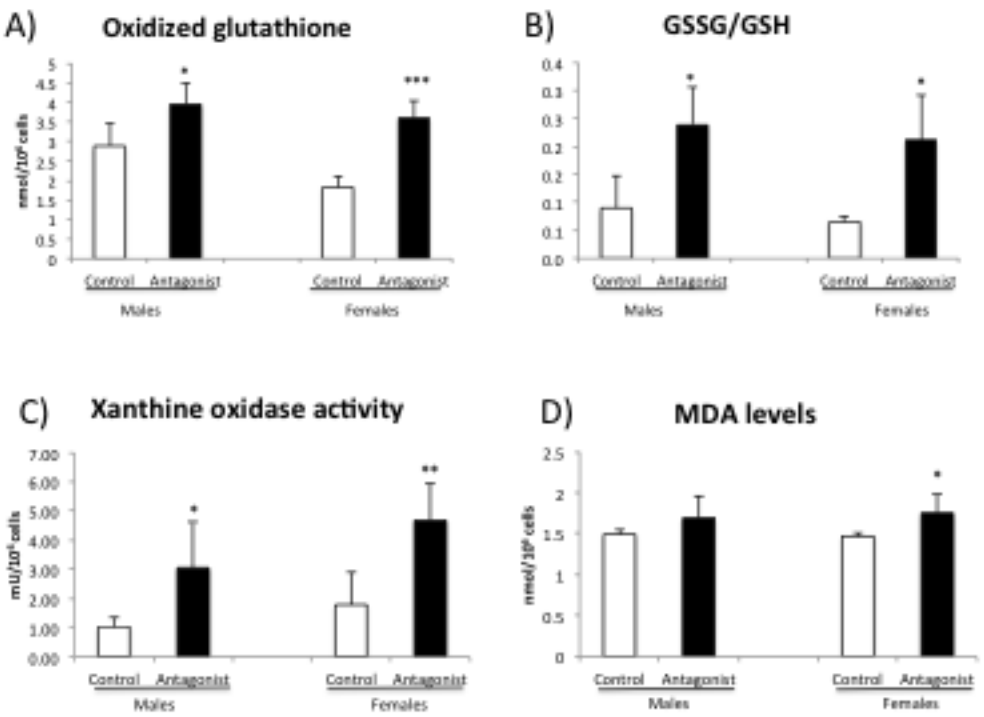
Figure 3. Antioxidants parameters. Reduced glutathione concentrations in adult male and female rats (A) treated neonatal with a leptin antagonist or vehicle (control). Glutathione peroxidase activity in adult male and female rats (B) treated neonatal with a leptin antagonist or vehicle (control). Glutathione reductase activity in adult male and female rats (C) treated neonatal with a leptin antagonist or vehicle (control). * $P<0.05$, ** $P<0.01$ with respect to the values of control rats.

Figure 4. Oxidative parameters. Oxidized glutathione concentrations in adult male and female rats (A) treated neonatal with a leptin antagonist or vehicle (control). GSSG/GSH ratios in adult male and female rats (B) treated neonatal with a leptin antagonist or vehicle (control). Xanthine oxidase activity in adult male and female rats (C) treated neonatal with a leptin antagonist or vehicle (control). MDA levels in adult male and female rats (D) treated neonatal with a leptin antagonist or vehicle (control). * $P<0.05$, ** $P<0.01$, ** $P<0.001$ with respect to the values of control rats.



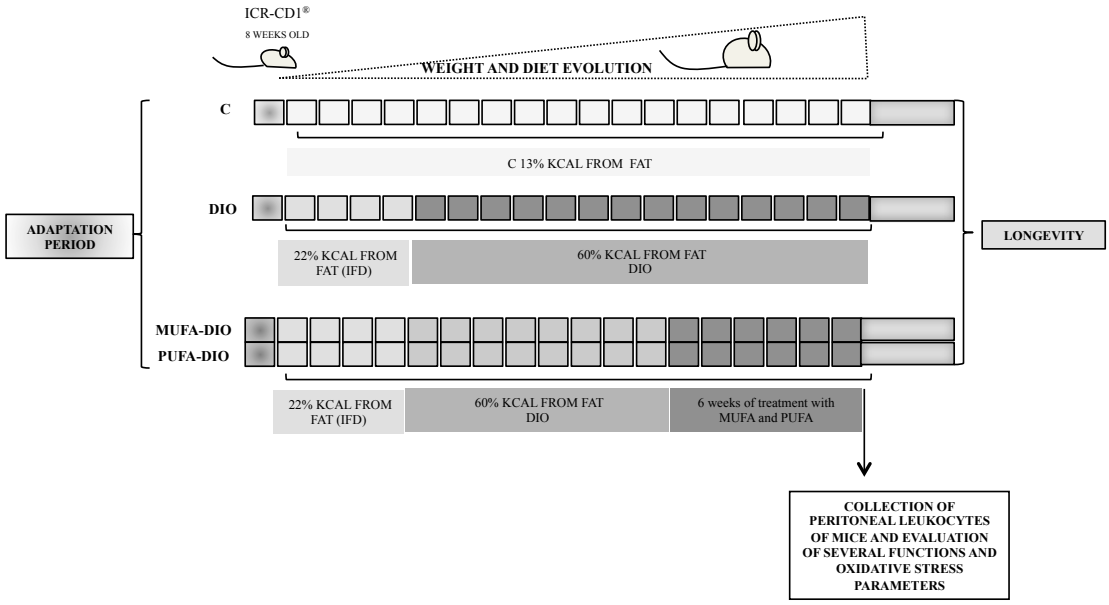






3.3. EFFECTS OF THE DIETARY SUPPLEMENTATIONS WITH 2-OHOA OR WITH THE COMBINATION OF N-3 FATTY ACIDS (EPA AND DHA) ON THE IMMUNE FUNCTION AND REDOX STATE OF ADULT DIET-INDUCED OBESE MICE, AS WELL AS ON THEIR LIFE SPAN

3.3.1. Effects of the dietary supplementations with 2-OHOA or with the combination of n-3 fatty acids (EPA and DHA) on the function and redox state of leukocytes of adult female diet-induced obese mice, as well as on their life span



Experimental design

During the first 5 days of acclimatization to the new environment, all mice were fed a standard maintenance diet (Teklad Global 14% Protein Rodent Maintenance Diet, reference 2014, Harlan Interfauna Iberica, with 13% Kcal from fat). At 9 weeks of age, the animals were separated into the following four groups (n=10, per group). 1) The control mice group (C) which was fed with a standard maintenance diet until the end of the study. 2) The diet-induced obese mice group (DIO) which was fed with a high-fat diet (HFD, 60% of calories from fat, reference TD. 06414, Harlan Interfauna Iberica) for 14 weeks. 3) The MUFA-supplemented DIO mice group (MUFA-DIO) which was fed with a high-fat diet for 14 weeks, but during the last 6 weeks of HFD, this diet was

supplemented with 2-hydroxyoleic acid (2-OHOA) (1500 mg of 2-OHOA per Kg of HFD, BTSA-Biotecnologías Aplicadas S.L.). 4) The n-3 PUFA-supplemented DIO mice group (PUFA-DIO) which was fed with a HFD for 14 weeks, this being supplemented during the last 6 weeks with the combination of EPA and DHA (3000 mg of EPA and DHA per Kg of HFD, BTSA-Biotecnologías Aplicadas S.L.). The 2-OHOA is a synthetic derivative of oleic acid, and it is also known as 2-hydroxy-D9-cis-octadecenoic acid. The n-3 PUFA were extracted from fish (anchovy). The fatty acid supplements (2-OHOA in powdered form and n-3 PUFA in oil form) were mixed with the chow, which was of malleable consistency, and then pelleted. In order to progressively increase the amount of fat in the diet of groups 2, 3 and 4, they were fed with an intermediate-fat diet (IFD, 22% of calories from fat, Teklad Global 2019, Harlan Interfauna Iberica) for 4 weeks previous to the ingestion of HFD. After receiving the HFD for 14 weeks, animals in groups 2, 3 and 4 returned to the standard maintenance diet until the end of the study, that is the say the natural death of the animals, which was monitored. During the entire study mice had free access to water and the diet. Weight and food intake were measured every week throughout the study.

Main results

Body weight and food intake measurements

All mice fed on a high-fat diet increased their body weight significantly in comparison with controls. After nutritional supplementation, when all mice received the standard maintenance diet, only DIO mice that were supplemented with MUFA significantly decreased their body weight when compared with DIO mice and with n-3 PUFA-DIO mice, having similar weights to those of controls. In addition, the three groups of DIO mice (DIO, MUFA-DIO and PUFA-DIO) fed a HFD, although ingesting a lower food intake, showed significantly higher quantities of calories consumed than animals fed a standard diet. However, when DIO mice supplemented with MUFA and n-3 PUFA or not, returned to the standard maintenance diet, no significant differences in food intake and total calories consumed were observed among all groups of mice.

Peritoneal leukocyte function parameters

The chemotaxis index of macrophages in response to a chemotactic gradient (formylated peptide) was significantly suppressed in DIO mice compared with controls. In addition, the number of latex beads ingested by macrophages, which is expressed as the phagocytic index, and the phagocytic efficiency were significantly lower in DIO mice with respect to controls. Nevertheless, the supplementations, with MUFA or with n-3 PUFA, in DIO mice were able to increase the chemotaxis index, the phagocytic index, and the phagocytic efficiency in comparison with non-supplemented DIO mice.

The values of intracellular superoxide anion in stimulated leukocytes (in the presence of latex beads), an indicator of the capacity of immune cells in killing pathogens in their phagosomes, were lower in DIO mice than in controls. The supplementation with the two types of dietary unsaturated fatty acids, MUFA or n-3 PUFA, resulted in higher concentrations of the superoxide anion than those of DIO mice.

The chemotaxis index of lymphocytes showed a significant decrease in DIO mice in comparison with control mice. The MUFA-DIO and PUFA-DIO groups of mice showed a significant increase when compared with non-supplemented DIO mice.

The NK activity against tumour cells was significantly suppressed in DIO mice as compared with that in non-obese control mice. However, the supplementation with n-3 FUFA tended to enhance this activity compared with that in non-supplemented DIO mice.

The lymphoproliferation in response to T-cell mitogen (ConA) and to B-cell mitogen (LPS) was highly suppressed in DIO mice with respect to non-obese control mice. The supplementations with MUFA and n-3 PUFA in DIO mice significantly increased the proliferation of lymphocytes in response to ConA and LPS when compared with non-supplemented DIO mice.

Peritoneal leukocyte oxidative stress parameters

The amount of total glutathione, which has an important antioxidant role, was decreased in leukocytes of DIO mice in comparison with those in non-obese controls. Mice that were

supplemented either with MUFA or with n-3 PUFA displayed significantly higher values of total glutathione than DIO animals.

The catalase activity was significantly diminished in DIO mice as compared with that in non-obese controls. The DIO mice that were supplemented with MUFA or with n-3 PUFA showed a significant increase in this antioxidant activity.

The activity of xanthine oxidase, which was significantly increased in DIO mice as compared with non-obese control mice, showed lower levels in DIO mice supplemented with n-3 PUFA when compared with those in non-supplemented DIO mice. Thus this supplementation decreases an activity associated with the production of free radicals.

The MDA concentrations, which are indicators of lipid oxidation and oxidative damage in cells, were significantly increased in DIO mice with respect to those in non-obese control mice. In turn, the supplementation with MUFA was able to diminish MDA concentrations in comparison with DIO mice.

Life span

The differences were not statistically significant among the groups of mice, however DIO mice exhibited a lower average survival (105 ± 13 weeks old) than controls (115 ± 15 weeks old). In addition, DIO mice supplemented with 2-OHOA displayed values similar to controls (110 ± 20 weeks old), whereas DIO mice supplemented with n-3 PUFA remained similar to DIO mice (105 ± 35 weeks old).

Partial conclusions

The supplementations with 2-OHOA or with the combination of n-3 PUFA (EPA and DHA) improve immune function and redox state of peritoneal leukocytes, which were impaired in DIO mice, bringing them to similar values to those in non-DIO controls.

Eur J Nutr
DOI 10.1007/s00394-017-1395-1

ORIGINAL CONTRIBUTION

Immune dysfunction and increased oxidative stress state in diet-induced obese mice are reverted by nutritional supplementation with monounsaturated and n-3 polyunsaturated fatty acids

Caroline Hunsche[1] · Oskarina Hernandez[1] · Alina Gheorghe[2] · Ligia Esperanza Díaz[2] · Ascensión Marcos[2] · Mónica De la Fuente[1]

Received: 20 July 2016 / Accepted: 2 February 2017

Abstract
Purpose Obesity is associated with impaired immune defences and chronic low levels of inflammation and oxidation. In addition, this condition may lead to premature aging. The aim of the study was to evaluate the effects of a nutritional supplementation with monounsaturated and n-3 polyunsaturated fatty acids on several functions and oxidative stress parameters in peritoneal immune cells of obese mice, as well as on the life span of these animals.
Methods Obesity was induced in adult female ICR/CD1 by the administration of a high-fat diet (HFD) for 14 weeks. During the last 6 weeks of HFD feeding, one group of obese mice received the same HFD, supplemented with 1500 mg of 2-hydroxyoleic acid (2-OHOA) and another with 3000 mg of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Several functions and oxidative stress parameters of peritoneal leukocytes were evaluated.
Results The groups of obese mice treated with 2-OHOA or with EPA and DHA showed a significant improvement in several functions such as chemotaxis, phagocytosis, digestion capacity, Natural killer activity and lymphoproliferation in response to mitogens. All of these functions, which were decreased in obese mice, increased reaching similar levels to those found in non-obese controls. Both treatments also improved oxidative stress parameters such as xanthine oxidase activity, which decreased, catalase activity and glutathione levels, which increased.
Conclusion These data suggest that dietary supplementation with monounsaturated and n-3 polyunsaturated fatty acids could be an effective nutritional intervention to restore the immune response and oxidative stress state, which are impaired in obese mice.

Keywords Obese mice · Monounsaturated fatty acids · n-3 Polyunsaturated fatty acids · Immune function · Oxidative stress

Introduction

Nutrition and dietary patterns have been shown to have a great impact on the functions of the immune system [1, 2]. Thus, as previously demonstrated in animal and human studies, the excessive consumption of energy-dense food, such as dietary fat, is associated with the development of obesity and dysfunctional immune response [3–7]. Although the precise mechanisms that underlie impaired immunity in obese individuals remain unclear, increasing evidence suggests that obesity and its related complications, including insulin resistance, type-2 diabetes mellitus and cardiovascular diseases, are linked to a state of chronic inflammation and oxidative stress [8, 9]. This condition seems to be generated and maintained by immune cell infiltration in the adipose tissue and its increased production of pro-inflammatory compounds and reactive oxygen species (ROS) [10, 11]. It is well known that inflammatory mediators and ROS have important physiological functions, such as those in immune response [12]. However, an excessive production of these compounds by immune cells

✉ Mónica De la Fuente
mondelaf@bio.ucm.es

[1] Department of Animal Physiology II Faculty of Biology, Complutense University of Madrid and Research Institute of Hospital 12 de Octubre (i+12), José Antonio Novais Street, 2, 28040 Madrid, Spain

[2] Department of Metabolism and Nutrition, Institute of Food Science, Technology and Nutrition (ICTAN), Spanish National Research Council (CSIC), José Antonio Novais Street, 10, 28040 Madrid, Spain

Published online: 23 February 2017

results in a vicious cycle of more inflammation, oxidation and detrimental effects, such as tissue damage and immune system dysfunction [11, 12]. Therefore, obese individuals have shown a worse immune response to bacterial and viral infections, and an increased prevalence of immune-related diseases [5, 6, 13]. Similar to obesity, the process of aging has been also associated with a less competent immune system. This immunosenescence results in a higher risk of age-related complaints [14]. In fact, it has recently been found that the development of obesity in experimental adult mice results in similar features of impaired immune system to those found in aged individuals, which suggests a premature aging of obese subjects [7, 15]. Therefore, the search for nutritional strategies that could ameliorate dysfunctional immunity in obese individuals and consequently promote a healthier aging seems important.

In this context, adequate amounts of certain kinds of nutrients have been proven to positively modulate immune cell functions. For instance, adequate antioxidant intake has been shown to maintain the antioxidant/oxidant balance of immune cells during aging, protecting these cells from age-related oxidative stress and immune dysfunction [16, 17]. Also, dietary unsaturated fatty acids have been suggested to promote a variety of health benefits, particularly in obesity [18, 19] and in the modulation of immune system functions [20–33]. Moreover, it is necessary to consider the quality of fats consumed. Thus, in contrast to the negative effects of the excessive intake of calories and dietary fat on immune functions, adequate amounts of unsaturated fatty acids that include monounsaturated (MUFA) of the n-9 series and polyunsaturated fatty acids of the n-3 series, seem to have beneficial effects on immune functions [20–34]. Thus, the n-9 MUFAs and n-3 PUFAs, i.e., eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which show anti-inflammatory actions, have been demonstrated as capable of modulating several functions of innate and adaptive immunity, including chemotaxis, phagocytosis of macrophages and generation of ROS in response to pathogens, anti-tumour NK cell activity and proliferation of B and T cells [20–33]. One of the possible mechanisms involved in these effects could be the incorporation of fatty acids into the immune cell membranes. This occurs after dietary lipid administration since it modulates cell functions, by modifying membrane fluidity, lipid peroxide formation, eicosanoid production and gene regulation [35]. In addition, a synthetic MUFA derivative of oleic acid, 2-hydroxyoleic acid (2-OHOA), has been found to induce body weight loss in rats [36] and in obese mice fed a high-fat diet [37]. Furthermore, this compound, at low doses and without apparent toxicity in animals [38], has shown to rapidly reduce arterial blood pressure when compared with natural oleic acid [39]. Thus, it is possible that this compound could also have immune benefits in obese individuals. Nevertheless, little is known about how supplementation with this oleic acid derivative or with n-3 fatty acids could affect immune cell functions and their oxidative stress state in obese subjects. Thus, the aim of this study was to evaluate several functions and parameters of oxidative stress in peritoneal leukocytes from high-fat diet-induced obese (DIO) mice supplemented with 2-OHOA, an oleic acid-derived compound, or with the combination of n-3 fatty acids (EPA and DHA). Since the parameters studied have been established as markers of health and longevity [14], the effects of these dietary supplementations on life span were also analysed.

Materials and methods

Animals

Female ICR/CD1 mice, 8 weeks of age, were purchased from Harlan Interfauna Iberica (Barcelona, Spain). The animals were housed in polyurethane cages (5 animals per cage) and maintained under standard laboratory conditions (12:12 h reversed light/dark cycle; lights on at 8:00 p.m., relative humidity of 50–60%, temperature of 22 ± 2 °C and adequate ventilation). The experiments were conducted in accordance with the guidelines and protocols of the Royal Decree 53/2013 regarding the care and use of laboratory animals for experimental procedures, and were approved by the Committee for Animal Experimentation of the Complutense University of Madrid.

Experimental groups

During the first 5 days of acclimatization to the new environment, all mice were fed a standard maintenance diet (Teklad Global 14% Protein Rodent Maintenance Diet, reference 2014, Harlan Interfauna Iberica, with 13% Kcal from fat). At 9 weeks of age, the animals were separated into four groups ($n = 10$, per group), namely (1) the control mice group (C) were fed with a standard maintenance diet until the end of the study; (2) the diet-induced obese mice group (DIO) were fed with a high-fat diet (HFD, 60% of calories from fat, reference TD. 06414, Harlan Interfauna Iberica) for 14 weeks; (3) The MUFA-supplemented DIO mice group (MUFA-DIO) were fed with a high-fat diet for 14 weeks, but during the last 6 weeks of HFD, this diet was supplemented with 2-OHOA (1500 mg of 2-OHOA per Kg of HFD, BTSA-Biotecnologías Aplicadas S.L.); (4) the n-3 PUFA-supplemented DIO mice group (PUFA-DIO) were fed with a HFD for 14 weeks, this being supplemented during the last 6 weeks with the combination of EPA and DHA (3000 mg of EPA and DHA per Kg of HFD, BTSA-Biotecnologías Aplicadas S.L.). To progressively increase the amount of fat in the diet of groups 2, 3 and 4, animals

of those groups were fed with an intermediate-fat diet (IFD, 22% of calories from fat, Teklad Global 2019, Harlan Interfauna Iberica) for 4 weeks previous to the ingestion of HFD. After receiving the HFD for 14 weeks, animals in groups 2, 3 and 4 were returned to the standard maintenance diet until the end of the study (the natural death of the animals, which mortality was monitored). This design is shown in Fig. 1. During the entire study, mice had free access to water and food. The full compositions of all the diets used are shown in Table 1. Weight and food intake were measured every week throughout the study. The doses of supplementation were calculated taking into consideration the daily average food intake of mice and the amount of supplemented 2-OHOA (1500 mg/kg) and EPA and DHA (3000 mg/kg) in the HFD (Table 2).

Collection of peritoneal leukocytes

The peritoneal suspensions were obtained between 8 a.m. and 10 a.m. to minimize circadian variations in the immune system, by a procedure previously described, without animal sacrifice [40, 41], which allowed monitoring the life span of the mice. Briefly, 3 ml of Hank's solution, adjusted to pH 7.4, was injected into the peritoneum, the abdomen was massaged and the peritoneal exudate cells were collected allowing the recovery of 90–95% of the injected volume. The peritoneal leukocytes, consisting of lymphocytes and macrophages, were counted in Neubauer chambers (Blau Brand, Germany). The suspensions were adjusted to a final concentration of 5×10^5 macrophages or lymphocytes/ml in Hank's solution or 10^6 leukocytes/ml in Hank's solution or complete medium (RPMI 1640 enriched with L-glutamine (PAA, Pasching, Austria) and supplemented with 10% heat-inactivated (56 °C, 30 min) fetal calf serum (GIBCO) and gentamicin (100 mg/ml, GIBCO) with or without phenol red, depending on the type of assay used). Macrophages and lymphocytes were identified by their morphology. The cellular viability was measured using the trypan-blue (Sigma, St Louis, MO, USA) exclusion test and in all cases was higher than 98%.

The immune functions and redox state parameters studied were performed using unfractionated peritoneal leukocytes, which better preserved the physiological environment surrounding the immune cells in vivo [42].

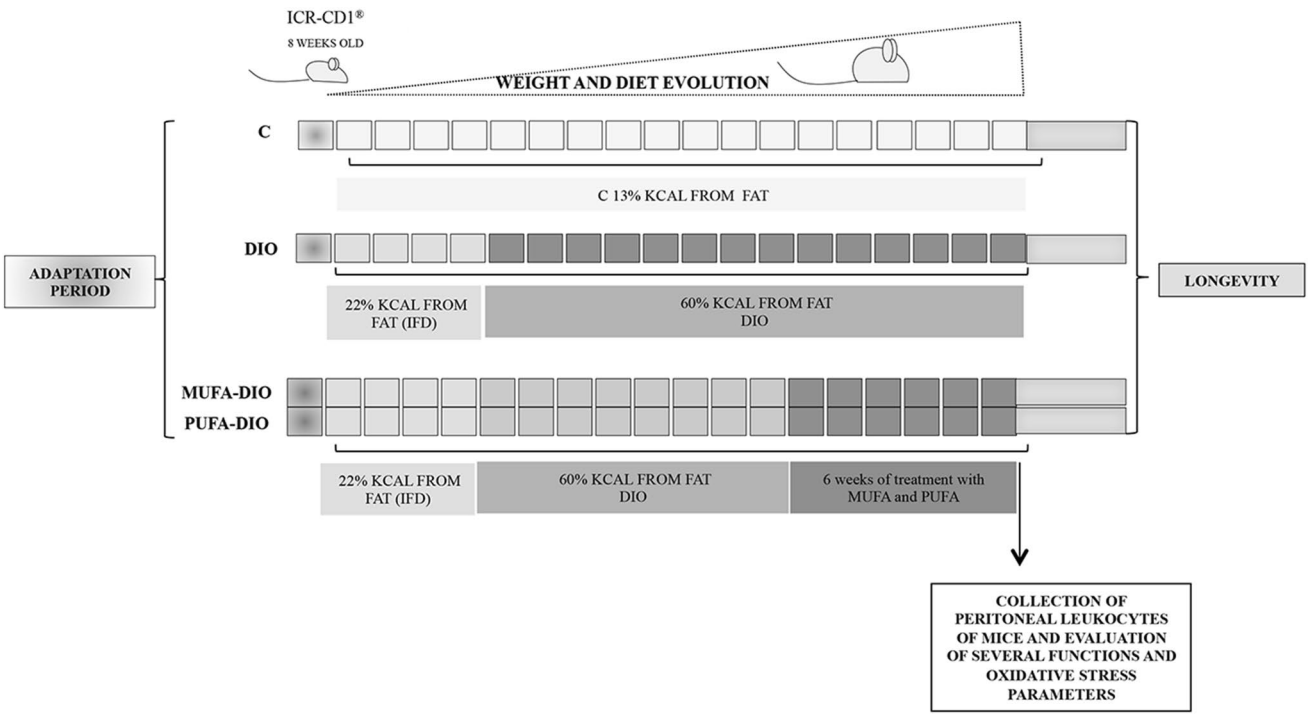


Fig. 1 Experimental design diagram. Four groups of mice are shown (1) *C* (control mice, fed with a standard maintenance diet until the end of the study with 13% of Kcal from fat); (2) *DIO* (diet-induced obese mice); (3) *MUFA-DIO* (DIO treated with monounsaturated fatty acid, MUFA); (4) *PUFA-DIO* (DIO treated with polyunsaturated fatty acids). Each *square* represents a period of 1 week. The *first square* corresponds to the adaptation period of mice in all groups. The DIO, MUFA-DIO and PUFA-DIO groups ingested a diet with 22% of kcal from fat (intermediate-fat diet: IFD) for 4 weeks. After this 4 weeks period, these groups received a diet 60% Kcal from fat (DIO) for 14 weeks. 6 weeks before the end of this period, mice of MUFA-DIO and PUFA-DIO groups ingested this DIO supplemented with MUFA and PUFAs. After that, peritoneal leukocyte samples were obtained and the animals in groups 2, 3 and 4 returned to the standard maintenance diet until the end of the study (the natural death of the animals, which mortality was monitored)

Table 1 Composition of the diets

Components	Standard maintenance diet	Intermediate-fat diet	High-fat diet	High-fat diet + MUFA supplementation	High-fat diet + PUFA supplementation
Energy (Kcal/g)	2.9	3.3	5.1	5.1	5.1
Protein (%)	14.3	19.0	23.5	23.5	23.5
Carbohydrate (%)	48.0	44.9	27.3	27.3	27.3
Fat (%)	4.0	9.0	34.3	34.4	34.6
Saturated (%)	0.6	1.2	12.5	12.5	12.5
Monounsaturated (%)	0.7	1.7	16.1	16.2	16.1
Polyunsaturated (%)	2.1	4.4	5.4	5.4	5.7
Protein (% of energy)	20.0	23.0	18.4	18.4	18.3
Carbohydrate (% of energy)	67.0	55.0	21.3	21.3	21.2
Fat (% of energy)	13.0	22.0	60.3	60.4	60.5
Fatty acid composition (%)					
C16:0 palmitic	0.5	0.9	8.2	8.2	8.2
C18:0 stearic	0.1	0.2	3.9	3.9	3.9
C18:1 oleic	0.7	1.7	14.7	14.8	14.7
C18:2 linoleic	2.0	3.9	4.7	4.7	4.7
C18:3 linolenic	0.1	0.4	0.5	0.5	0.8
n-6: n-3 ratio	20:1	10:1	9:1	9:1	6:1

Table 2 Body weight, food intake and supplementation doses

	Control mice	DIO mice	MUFA-DIO mice	PUFA-DIO mice
Average weight (g/mouse)				
HFD period (14–22 weeks old)	32 ± 0.7	39 ± 2.3***	38 ± 2.1***	40 ± 2.3***
HFD + supplementation period (22–28 weeks old)	33 ± 0.7	44 ± 1***	43 ± 1.1***	43 ± 0.9***
Standard maintenance diet period (42–72 weeks old)	38 ± 1.7	45 ± 3.1***	36 ± 2.4$^{###}$	46 ± 3.6***, $^{\$\$\$}$
Average food intake				
HFD period (14–22 weeks old) (g/mouse/day)	4.2 ± 0.6	2.8 ± 0.4***	3.2 ± 0.8***	2.8 ± 0.4***
HFD period (14–22 weeks old) (Kcal/mouse/day)	12 ± 1.7	14 ± 1.9*	16 ± 4.0***	14 ± 2.2*
HFD + supplementation period (22–28 weeks old) (g/mouse/day)	4 ± 0.6	3.2 ± 0.8*	3 ± 0.5**	3.1 ± 0.6*
HFD + supplementation period (22–28 weeks old) (Kcal/mouse/day)	11 ± 1.9	17 ± 4.4**	15 ± 2.9*	16 ± 3.2**
Standard maintenance diet period (42–72 weeks old) (g/mouse/day)	4.3 ± 0.5	4.1 ± 0.6	4.2 ± 0.6	4.2 ± 0.9
Standard maintenance diet period (42–72 weeks old) (Kcal/mouse/day)	12 ± 1.6	12 ± 2.4	12 ± 1.8	12 ± 2.6
Supplementation doses				
2-OHOA (mg/mouse)			4.5	
EPA + DHA (mg/mouse)				9.3

HFD high-fat diet, *DIO* diet-induced obese mice, *MUFA* monounsaturated fatty acids, *PUFA* polyunsaturated fatty acids, 2OHOA 2-hydroxy-oleic acid, EPA + DHA eicosapentaenoic acid + docosahexaenoic acid, *weeks* weeks

Each value represents the mean ± *SD* of the data obtained weekly during the period indicated corresponding to 8–10 animals

***$P < 0.001$

**$P < 0.01$; $P < 0.05$ with respect to the values of control mice

$^{###}P < 0.001$ with respect to the values of DIO mice

$^{\$\$\$}P < 0.001$ with respect to the values of MUFA-DIO mice

Chemotaxis assay

Chemotaxis of peritoneal leukocytes was evaluated according to a slight modification of Boyden´s method [40], consisting basically of the use of chambers with two compartments separated by a filter with a pore diameter of 3 µm (Millipore, Bedford, MA, USA). Aliquots of 300 µl of the peritoneal suspensions, with macrophages or lymphocytes adjusted to 5×10^5 cells/ml in Hank's solution, were deposited in the upper compartment, and aliquots of 400 µl of the chemoattractant, formyl–Met–Leu–Phe (10^{-8} M) (Sigma, St Louis, MO, USA), were put into the lower compartment. The chambers were incubated for 3h and then the filters were fixed and stained. The chemotaxis index (CI) was determined by counting, in an optical microscope (100×), the total number of macrophages and lymphocytes on one-third of the lower face of the filters.

Phagocytosis assay

Phagocytosis assay of inert particles (latex beads) was carried out following a method previously described [40]. Aliquots of 200 µl of the peritoneal suspensions adjusted to 5×10^5 macrophages/ml in Hank's medium were incubated in migration inhibitory factor (MIF) plates (Kartell, Noviglio, Italy) for 30 min. The adhered monolayer was washed with pre-warmed PBS (phosphate buffer saline), and then 200 µl of Hank's medium and 20 µl of latex bead suspension (1.09 µm, diluted to 1% PBS) (Sigma, St Louis, MO, USA) were added. After 30 min of incubation, the plates were washed, fixed and stained. The number of particles ingested by 100 macrophages was counted using an optical microscope (100×) and expressed as phagocytic index (PI). The percentage of macrophages, which phagocyted at least one latex bead, was also determined and expressed as phagocytic efficiency (PE).

Superoxide anion level assay

The superoxide anion level was evaluated assessing its capacity to reduce nitroblue tetrazolium (NBT, Sigma), in an equimolecular reaction, following the method previously described [40]. Briefly, aliquots of 250 µl of peritoneal cell suspensions (1×10^6 leukocytes/ml Hank's medium) were mixed with 250 µl of NBT solution (1 mg/ml in Hank's solution) (Sigma, St Louis, MO, USA) and with 50 µl of latex bead suspension (1%) in stimulated samples. After 60 min of incubation at 37 °C, the reaction was stopped, the samples were centrifuged, and the supernatants discarded. The reduced NBT was extracted with dioxin (Merck, Darmstadt, FRG) and the absorbance of the supernatants was determined at 525 nm using a spectrophotometer. The data obtained were expressed as nmoles of NBT reduced per 10^6 leukocytes by extrapolating in a standard curve of NBT reduced with 1,4-dithoerythritol (Sigma, St Louis, MO, USA).

Natural killer assay

An enzymatic colorimetric assay was carried out to measure the cytolysis of tumour cells (Cytotox 96 TM Promega, Boehringer, Ingelheim) based on the determination of lactate dehydrogenase enzyme (LDH), as previously described [40]. Aliquots of 100 µl of peritoneal leukocytes, used as effector cells, were seeded in 96-well U-bottom culture plates (Numc, Roskilde, Denmark) adjusted to 10^6 leukocytes per well in RPMI 1640 medium without phenol red. Murine lymphoma YAC-1 cells, used as target cells, were added adjusted to 10^5 cells per well. Thus, the effector/target ratio was 10:1. The plates were centrifuged at 250 g for 4 min to facilitate cell contacts. After 4 h of incubation, lactate dehydrogenase enzymatic activity was measured in 50 µl/well of the supernatants by addition of the enzyme substrate and absorbance recording spectrophotometrically at 490 nm. Three kinds of control measurements were performed: a target spontaneous release, a target maximum release, and an effector spontaneous release. The results were expressed as percentage of lysis of target cells. To determine this percentage the following equation was used: $\%\text{lysis} = [(E-ES-TS)/M-ES-TS)]$ x 100, where E is the mean of absorbance in the presence of effector cells; ES, the mean of absorbance of effector cells incubated alone; TS, the mean of absorbance in target cells incubated with medium alone; and M is the mean of maximum absorbance after incubating target cells with lysis solution.

Lymphoproliferation assay

Following the method previously described [41], aliquots (200 µl) of peritoneal leukocytes (10^6 cells/ml complete medium) were seeded in 96 well flat-bottomed microtitre plates (Numc, Roskilde, Denmark) and 20 µl of concanavaline A (ConA 1 µg/ml; Sigma, St Louis, MO), 20 µl of lipopolysaccharide (LPS, *E. coli*, 055:B5 1 µg/ml; Sigma) or 20 µl of complete medium (spontaneous proliferation) were added per well. After 48h of incubation at 37 °C in an atmosphere of 5% CO2, 0.5 µCi ^{3}H-thymidine (Du Pont, Boston, MA, USA) was added to each well. The cells were harvested in a semiautomatic microharvester, 24h later. Thymidine uptake was measured using a beta counter (LKB, Uppsala, Sweden). The results were expressed as ^{3}H-thymidine uptake (cpm).

Total glutathione levels assay

The total intracellular glutathione levels were measured using the Tietze enzymatic assay, with some modifications [43]. Briefly, aliquots of 1 ml of peritoneal suspension (adjusted to 10^6 leukocytes per ml in Hank's medium) were centrifuged at 1200*g* for 10 min at 4 °C. The pellet cells were re-suspended in a medium containing 5% tricloroacetic acid (TCA, Panreac, Barcelona, Spain) in 0.01 N HCl (previously degassed with helium for a minimum of 10 min). This was followed by three cycles of sonication for 10 s (with 20 s rest between each cycle), keeping the sample cold. Then, the samples were centrifuged at 3200*g* for 5 min at 4 °C. Aliquots of the supernatants of leukocytes samples were measured using the following reaction mixture: 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB, 6 mM, Sigma), β-nicotinamide adenine dinucleotide phosphate, reduced form (β-NADPH, 0.3 mM, Sigma), and glutathione reductase (10 U/ml, Sigma). The reaction was monitored for 240 s and measured using spectrophotometry at a wavelength of 412 nm. The results were expressed in nmoles/10^6 cells.

Catalase activity assay

The activity of catalase (CAT) was determined following the method described by Beers and Sizer, with slight modifications [16]. The peritoneal suspension was previously adjusted to 10^6 leukocytes/ml and aliquots of 1 ml were used to perform the enzymatic assay. The cells were centrifuged at 1076*g* for 10 min at 4 °C and the pellets were re-suspended in 50 mM phosphate buffer, containing 14 mM of H_2O_2 (Merck, Germany). Then, the samples were sonicated and centrifuged at 3200*g* for 20 min at 4 °C. The enzymatic assay was followed using spectrophotometry for 80 s at 240 nm. The results were expressed as international units (U) of enzymatic activity per 10^6 cells.

Xanthine oxidase activity assay

Xanthine oxidase (XO) activity was measured by fluorescence using a commercial kit "Amplex Red Xanthine/Xanthine Oxidase Assay Kit" (Molecular Probes, Paisley, UK), as previously described [44]. The hydrogen peroxide (H_2O_2) produced by XO reacts with the horseradish peroxidase present in the reaction mixture and generates a fluorescent oxidation compound resorufin whose fluorescence is measured in a plate reader (Fluorestar Optima, BMG Labtech Biomedal, Spain). Briefly, 50 µl of peritoneal suspension adjusted to 10^6 leukocytes/ml in Hank's medium was incubated with 50 µl working solution of Amplex Red reagent (100 µM) containing HRP (0.4 U/ml) and xanthine (200 µM). After 30 min of incubation at 37 °C, measurements of fluorescence was performed in a microplate reader using excitation at 530 nm and emission detection at 595 nm. Data analysis was performed with xanthine standard curves at different concentrations, the results being expressed in international milliunits (mU) of enzymatic activity per 10^6 cells.

Lipid peroxidation (MDA) assay

The estimation of malondialdehyde (MDA) in cells was evaluated using the commercial kit "MDA Assay Kit" (Biovision, Mountain View, CA, USA), which measures the reaction of MDA with thiobarbituric acid (TBA) and the MDA-TBA adduct formation. Aliquots of 1 ml of leukocytes adjusted to 10^6 cells/ml of Hank's were centrifuged at 1200 g for 10 min at 4 °C and the pellets were re-suspended in 300 µl of MDA lysis buffer with 3 µl BHT. Then, the cell suspensions were sonicated and centrifuged at 13,000*g* for 10 min at 4 °C to remove insoluble material. The supernatant (200 µl) from each sample was added to 600 µl of TBA reagent. After the incubation at 95 °C for 60 min, samples were cooled to room temperature in an ice bath for 10 min and then 200 µl of supernatant was dispensed into a 96-well microplate. The MDA-TBA adduct was measured using a spectrophotometer at 532 nm of absorbance.

Statistical analysis

SPSS 10.0 (SPSS, Inc., Chicago, IL) was used for the statistical analysis of the results. The data were expressed as mean ± standard deviation (SD). Each value is the mean of the data from an assay performed in duplicate or triplicate. Normality of the samples was checked by the Kolmogorov–Smirnov test and homogeneity of variances with the Levene test. The data were statistically evaluated by one-way ANOVA followed by Tukey's post hoc test for homogenous variances, whereas Games–Howell´s post hoc test was used for unequal variances. Finally, a Kaplan–Meier survival analysis was conducted to verify the effects of both MUFA and n-3 PUFA treatments on the survival of DIO mice. $P < 0.05$ was considered statistically significant and $0.05 < P < 0.1$ as a trend.

Results

Body weight and food intake

As shown in Table 2, all mice fed on a high-fat diet increased their body weight significantly in comparison with controls ($P < 0.001$). After nutritional supplementation, when all mice received the standard maintenance

diet, only DIO mice that were supplemented with MUFA significantly decreased their body weight when compared with DIO mice and with n-3 PUFA-DIO mice ($P<0.001$), having similar weights to those of controls. In addition, the three groups of DIO mice (DIO, MUFA-DIO and PUFA-DIO) fed a HFD, although ingesting a lower food intake, showed significantly higher values of calories consumed than animals that fed a standard diet ($P<0.05$, $P<0.001$ and $P<0.05$, respectively; Table 2). However, when DIO mice supplemented with MUFA and n-3 PUFA or not, returned to the standard maintenance diet, no significant differences in food intake and total calories consumed were observed among all groups of mice (Table 2).

Macrophage peritoneal functions

The peritoneal macrophage functions are shown in Fig. 2. The chemotaxis index of macrophages in response to a chemotactic gradient (formylated peptide), which mimics the migration of immune cells towards the infection site, was significantly suppressed in DIO mice compared with controls ($P<0.01$; Fig. 2a). In addition, the number of latex beads ingested by macrophages, which is measured by the phagocytic index ($P<0.01$; Fig. 2b), and the phagocytic efficiency ($P<0.01$; Fig. 2c) were significantly lower in DIO mice with respect to controls. Nevertheless, the supplementations, with MUFA or with n-3 PUFA, in DIO mice were able to increase the chemotaxis index ($P<0.01$ and $P<0.01$, respectively; Fig. 2a), the phagocytic index ($P<0.001$ and $P<0.05$, respectively; Fig. 2b) and the phagocytic efficiency ($P<0.05$ and $P=0.06$, respectively; Fig. 2c) in comparison with non-supplemented DIO mice.

The levels of intracellular superoxide anion in stimulated leukocytes (in the presence of latex beads), an important capacity of immune cells in killing pathogens

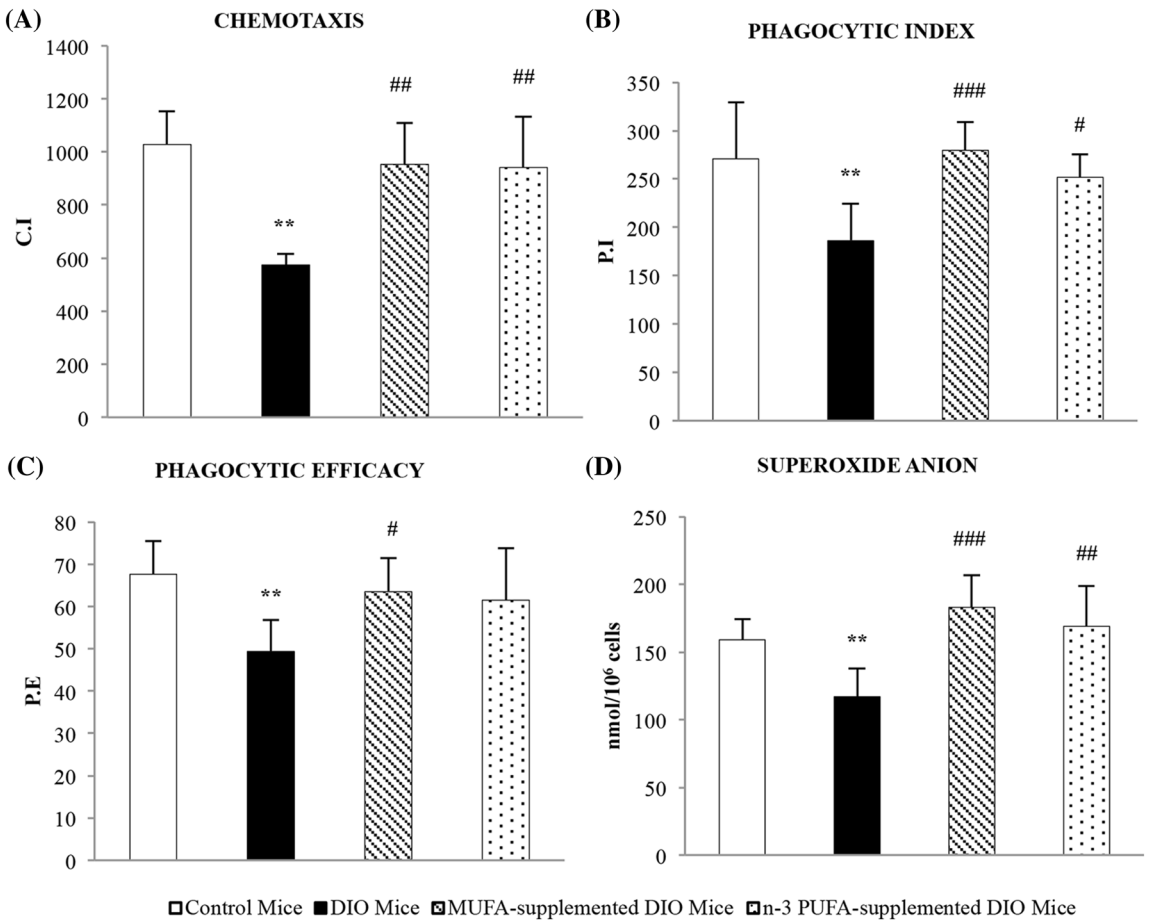


Fig. 2 Macrophage functions. **a** Macrophage chemotaxis index (*CI*, number of macrophages). **b** Macrophage phagocytic index (*PI*, number latex beads/100 macrophages). **c** Macrophage phagocytic efficacy (*PE*, number of phagocyting macrophages/100 macrophages). **d** Stimulated intracellular superoxide anion levels (nmol/10^6 cells). *Each column* represents the mean ± SD of 8–10 values corresponding to that number of animals and each value being the mean of duplicate or triplicate assays. $^{***}P<0.001$; $^{**}P<0.01$ with respect to the values of non-obese control mice. $^{\#\#\#}P<0.001$; $^{\#\#}P<0.01$; $^{\#}P<0.05$ with respect to the values of DIO mice

in their phagosomes, showed significantly lower values in DIO mice ($P<0.01$) when compared with controls (Fig. 2d). The supplementation with the two types of dietary unsaturated fatty acids, MUFA or n-3 PUFA, resulted in higher levels of the superoxide anion than those of DIO mice ($P<0.001$ and $P<0.01$, respectively; Fig. 2d).

Lymphocyte peritoneal functions

The lymphocyte functions are displayed in Fig. 3. The chemotaxis index induced by a chemotactic peptide (Fig. 3a) showed a significant decrease in DIO mice in comparison with control mice ($P<0.001$). The MUFA-DIO and PUFA-DIO groups of mice showed a significant increase when compared with non-supplemented DIO mice ($P<0.01$; Fig. 3a).

The NK cell activity against tumour cells (Fig. 3b) was significantly suppressed in DIO mice as compared with that in non-obese control mice ($P<0.05$). However, the supplementation with n-3 FUFA tended to enhance this activity compared with that in non-supplemented DIO mice ($P=0.08$).

The lymphoproliferation in response to T-cell mitogen (ConA) and to B-cell mitogen (LPS) (Fig. 3c, d) was highly suppressed in DIO mice with respect to non-obese control mice ($P<0.001$). The supplementation with MUFA and n-3 PUFA in DIO mice significantly increased the proliferation of lymphocytes in response to ConA (Fig. 3c) and LPS (Fig. 3d) when compared with non-supplemented DIO mice ($P<0.05$ and $P<0.01$, respectively, for ConA and $P<0.05$ and $P<0.001$, respectively, for LPS).

Peritoneal leukocyte oxidative stress parameters

Figure 4 shows oxidative stress parameters of peritoneal leukocytes. The levels of total glutathione (Fig. 4a), which have an important antioxidant role, were decreased in leukocytes of DIO mice in comparison with those in non-obese controls ($P<0.001$). Mice that were supplemented either with MUFA or with n-3 PUFA displayed

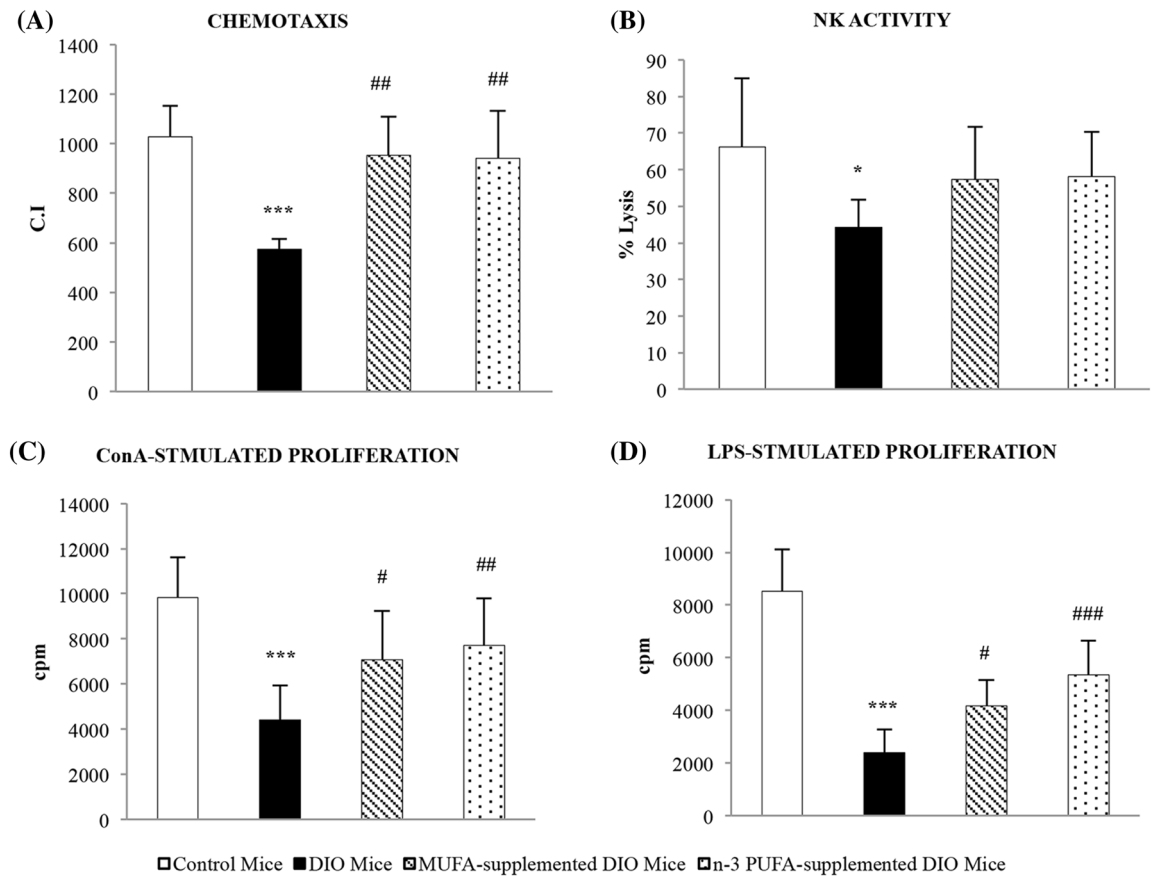


Fig. 3 Lymphocyte functions. **a** Lymphocyte CI (number of lymphocytes). **b** Natural killer cell activity (% lysis). **c** Lymphoproliferative response to concanalvaline A (ConA) (cpm). **d** Lymphoproliferative response to lipopolysaccharide (LPS) (cpm). $^{***}P<0.001$; $^{**}P<0.01$; $^{*}P<0.05$ with respect to the values of non-obese control mice. $^{\#\#\#}P<0.001$; $^{\#\#}P<0.01$; $^{\#}P<0.05$ with respect to the values of DIO mice

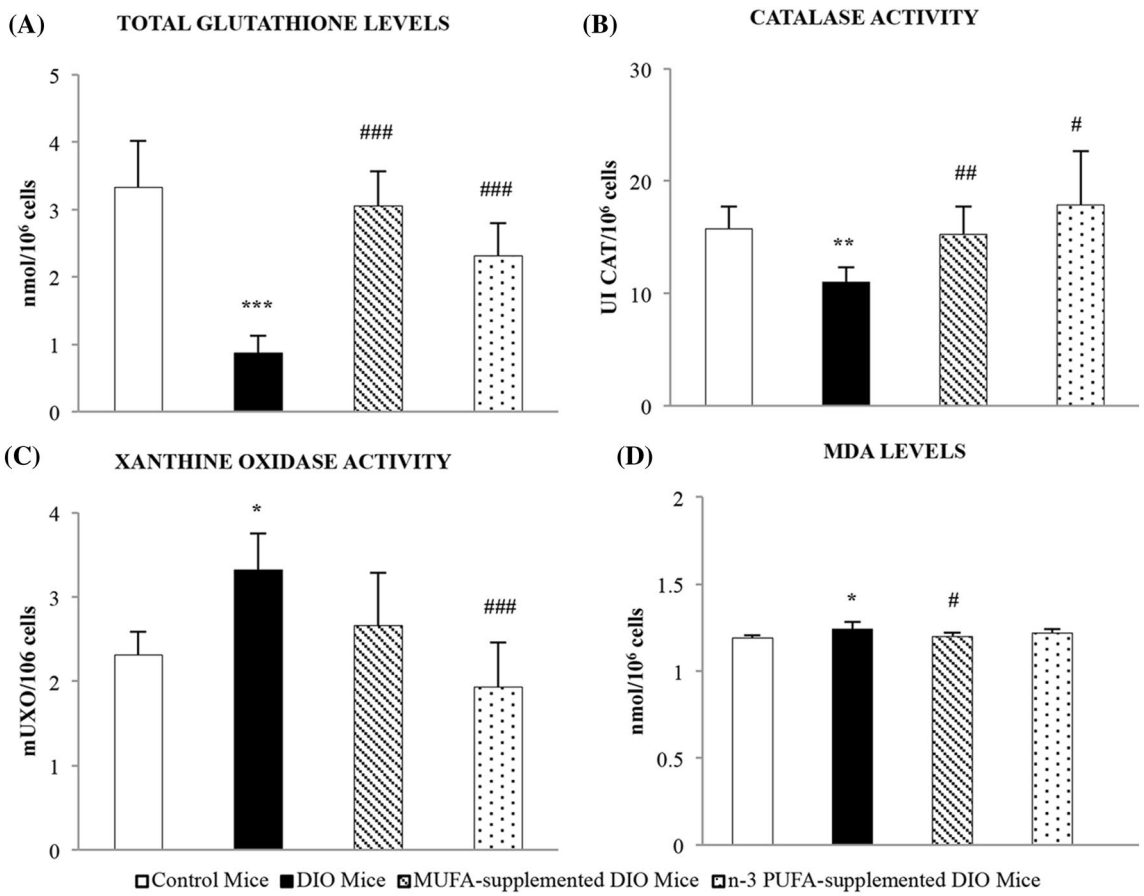


Fig. 4 Oxidative stress parameters. **a** Total glutathione levels (nmol/10^6 cells). **b** Catalase activity (CAT, U CAT/10^6 cells). **c** Xanthine oxidase activity (mUXO/10^6 cells). **d** Malondialdehyde (MDA) levels (nmol/10^6 cells). $^{***}P<0.001$; $^{*}P<0.05$ with respect to the values of non-obese control mice. $^{\#\#\#}P<0.001$; $^{\#\#}P<0.01$; $^{\#}P<0.05$ with respect to the values of DIO mice

significantly higher values of total glutathione than DIO animals ($P<0.001$; Fig. 4a).

The catalase activity (Fig. 4b), an antioxidant enzyme, was significantly diminished in DIO mice as compared with that in non-obese controls ($P<0.01$). The DIO mice that were supplemented with MUFA or with n-3 PUFA showed a significant increase in catalase activity ($P<0.01$ and $P<0.05$, respectively; Fig. 4b).

The activity of xanthine oxidase (Fig. 4c), which is associated with the production of free radicals, was significantly increased in DIO mice as compared with non-obese control mice ($P<0.05$). DIO mice supplemented with n-3 PUFA showed lower levels of xanthine oxidase when compared with those in non-supplemented DIO mice ($P<0.001$).

The MDA levels (Fig. 4d), which are an indicator of lipid oxidation and oxidative damage in cells, were significantly increased in DIO mice with respect to those in non-obese control mice ($P<0.05$). In turn, the supplementation with MUFA was able to diminish MDA levels in comparison with DIO mice ($P<0.05$).

Life span

The differences were not statistically significant among the groups of mice; however, DIO mice exhibited a lower average survival (105 ± 13 weeks old) than controls (115 ± 15 weeks old). In addition, DIO mice supplemented with 2-OHOA displayed values similar to controls (110 ± 20 weeks old), whereas DIO mice supplemented with n-3 PUFA remained similar to DIO mice (105 ± 35 weeks old) (Fig. 5).

Discussion

Previous results, in which a similar experimental design was used, have demonstrated that the ingestion of a high-fat diet during early ages produces obesity and dysfunctional immunity in adulthood. Animals fed on a high-fat diet during 14 weeks of their adolescence showed elevated levels of triglycerides and systolic arterial pressure, which are common features of obesity [7, 45]. The parameters of

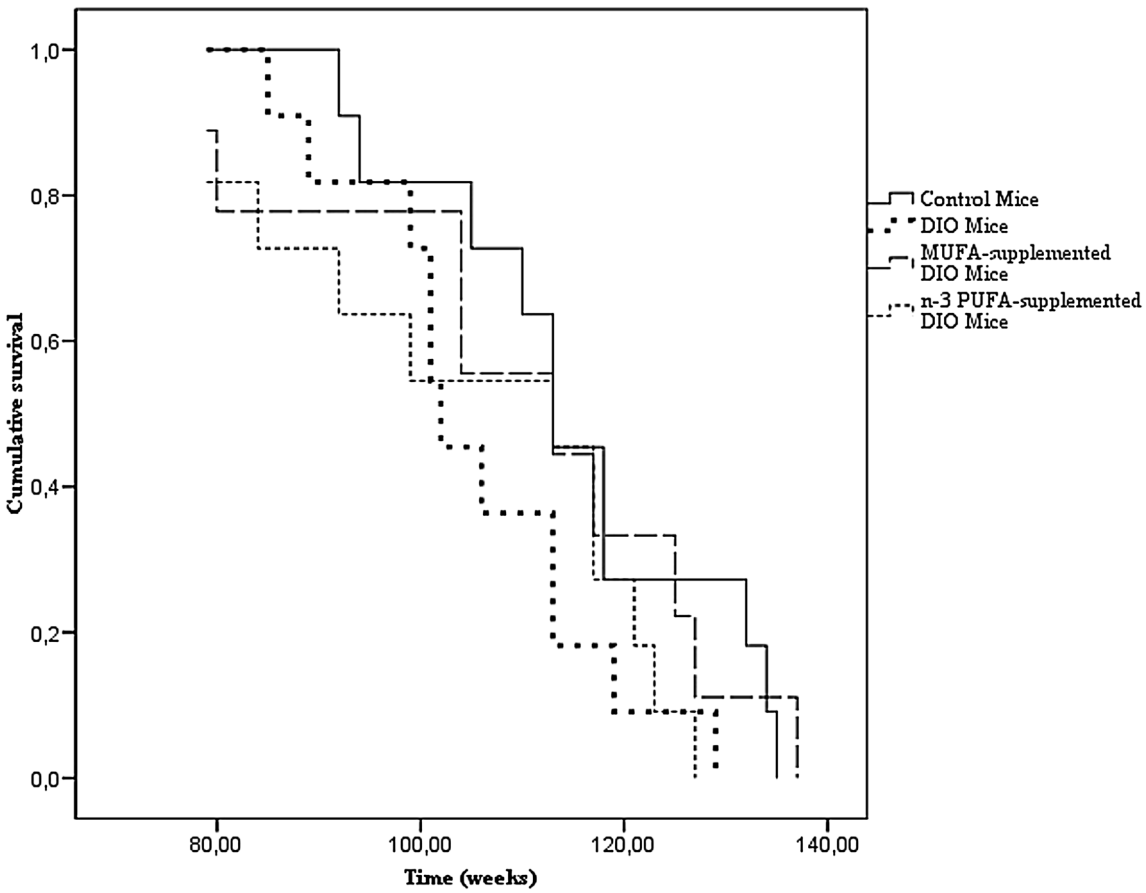


Fig. 5 Mortality records from control (ICR/CD1 female) mice and from DIO (diet-induced obese) mice, as well as from DIO treated with monounsaturated fatty acid (MUFA-DIO) or with polyunsaturated fatty acid (PUFA-DIO) mice

function and redox state of peritoneal leukocytes evaluated in the current study have been established as markers of health and rate of aging [14]. Thus, the adult diet-induced obese mice presented some characteristics of premature aging, such as premature immunosenescence. Moreover, these adult obese animals reached old age in a worsened condition, in spite of having returned to a control diet during their aging process. Hence, they continued to exhibit higher impairments of immune cell functions and consequently a shorter life span than the non-obese animals [7]. Because of the deleterious and lasting effects of obesity on immune cell functions, we investigated whether the supplementation with dietary unsaturated fatty acids (MUFA and n-3 PUFA), which are known for their immune-modulatory action, could improve the immune response and redox state of peritoneal leukocytes of mice, as well as their life span.

The results of the present study found that the dietary supplementations with 2-OHOA or with the combination of EPA and DHA were able to restore several immune cell functions and redox state of leukocytes, which were impaired in DIO mice. In addition, a long-lasting decrease in the body weight of 2-OHOA supplemented-DIO mice was observed during their aging process. Similarly, another study also found that 2-OHOA-treated rats experience a decrease in body weight through reduction of adipose fat mass [36]. The beneficial effects of 2-OHOA were also reflected in the life span of mice, and thus, although without statistical significance, the treated mice displayed an average survival (number of weeks) similar to control mice. Nevertheless, n-3 PUFA supplemented-DIO mice only showed significant improvements in immune and oxidative stress parameters, but no decrease in body weight or increase in life span. In that sense, evidence from a recent meta-analysis also indicates that supplementation with n-3 PUFA does not promote anti-obesity effects, such as reduction of weight, in overweight/obese individuals [46].

The dietary supplementation with 2-OHOA or with the combination of EPA and DHA was able to revert the impaired macrophage functions of DIO mice, indicating a boost of the innate immune response towards pathogen destruction. Thus, DIO-treated mice displayed improved chemotaxis, phagocytosis and generation of stimulated superoxide anion in peritoneal leukocytes. In agreement, findings from in vitro studies showed that neutrophils

incubated with MUFA or with n-3 PUFA presented enhanced migration, phagocytic capacity and ROS production by immune cells, suggesting immune-enhancing properties of fatty acids [20–23]. Several studies demonstrated that EPA and DHA could modulate immune functions in neutrophils of rats and humans, increasing chemotactic response, phagocytic activity and ROS production [24, 25]. In addition, administration of oleic acid induced neutrophil recruitment in a rat air-pouch model [26] and mice that were fed a diet enriched with olive oil (MUFA) exhibited increased phagocytic activity in peritoneal macrophages [27].

The NK cell activity from peritoneal immune cells, which has also been shown to be impaired in DIO mice [3, 7, 15], tended to be higher after supplementation with n-3 PUFA. Previous work has demonstrated that ingestion of n-3 PUFA is able to improve NK cell cytotoxicity in sedentary rats [28]. However, supplementation with MUFA did not lead to significant differences in the NK cell activity of DIO mice. Similarly, another study also did not find differences in the activity of NK cells in middle-aged men supplemented with MUFA-rich diet [29].

The lymphocyte functions, such as migration and proliferation in response to T cell-specific mitogen (ConA) or B cell-specific mitogen (LPS), showed lower values in DIO mice than in non-obese mice. However, the dietary supplementation used led to higher values for these functions. In this sense, an increased resistance to infection was found associated with enhanced proliferation of peritoneal cells under LPS stimulation in mice fed fish oil, rich in EPA and DHA, during 6 weeks [30]. An improvement in the production of circulating IgM in response to an antigen was obtained after administration of a n-3 PUFA-rich diet in obese mice [31]. Another study, in which mice were fed an olive oil-rich diet, exhibited slightly increased splenic lymphocyte proliferation [32]. Nevertheless, some animal studies have described an inhibition of lymphocyte proliferation with MUFA supplementation [33], whereas human studies have shown no changes [29]. It is possible that these contradictory results could be influenced by the doses supplemented in the diets. A study in this regard found that lower doses of oleic acid enhanced lymphocyte proliferation, while higher concentrations could result in inhibition of proliferation through an increase in apoptosis [47].

Oxidative stress, which is generated by an imbalance between antioxidants and oxidants, also seems to play a critical role in obesity and obesity-related diseases [48]. In fact, in this study, DIO mice showed higher values of oxidant production, such as xanthine oxidase activity and lipid peroxidation (MDA), as well as lower values of antioxidants (catalase activity and total glutathione levels) than non-obese control mice. This indicates that these mice seemed to exhibit an augmented oxidative stress state in comparison with controls. However, the dietary supplementation with MUFA or n-3 PUFA seems to enhance endogenous antioxidant defences in immune cells, as both treatments have resulted in increased catalase activity and GSH levels. In addition, n-3 PUFA supplementation led to a lower activity of xanthine oxidase, whereas dietary supplementation with MUFA, resulted in a diminished production of MDA in peritoneal immune cells. Thus, these findings suggest that EPA plus DHA as well as 2-OHOA have a role in improving redox state in obese mice. Moreover, 2-OHOA appears to be less susceptible to lipid peroxidation than n-3 fatty acids, since supplementation with n-3 did not significantly diminish MDA levels. In agreement, previous studies described that the supplementation with extra virgin olive oil, which has been attributed an antioxidant role mostly by its high content of oleic acid [19], diminished the lipid peroxidation in rats [49, 50]. Supplementation with n-3 PUFA also exhibited antioxidant properties in rats [51] and in a mouse model [52].

In conclusion, the supplementation with 2-OHOA or n-3 PUFA (EPA and DHA) improved several function and oxidative stress parameters of impaired leukocytes in obese mice, bringing the values to similar levels as those found in non-obese controls. The results suggest that these nutritional treatments could be a promising strategy to improve functions of the immune system and to decrease the oxidative state in obese individuals. Moreover, given that an improved immune system is associated with a diminishment of oxidation and inflammation states [7, 14, 17], it could also be related to an amelioration of the pathophysiology of obesity and its related diseases, thus, ensuring better health in adulthood and during the aging process.

Author contributions MDF, AM and LED contributed to the study design; CH, OH and AG conducted the experiments; CH, OH and AG performed the data analysis; CH and MDF wrote the paper. All authors read and approved the final manuscript.

Acknowledgements This work was supported by grants from Ministry of Science and Innovation (BFU2011-30336), the Research Group of Madrid Complutense University (910379ENEROINN), Red Temática de Investigación Cooperativa en Envejecimiento y Fragilidad (RETICEF RD12/0043/0018) and FIS (PI15/01787) of Institute de Salud Carlos III—Fondo Europeo de Desarrollo Regional (ISCIII-FEDER), the PRONAOS study and BTSA-Applied Biotechnologies S.L. Caroline Hunsche is the recipient of a PhD fellowship from CNP-q-Brazil.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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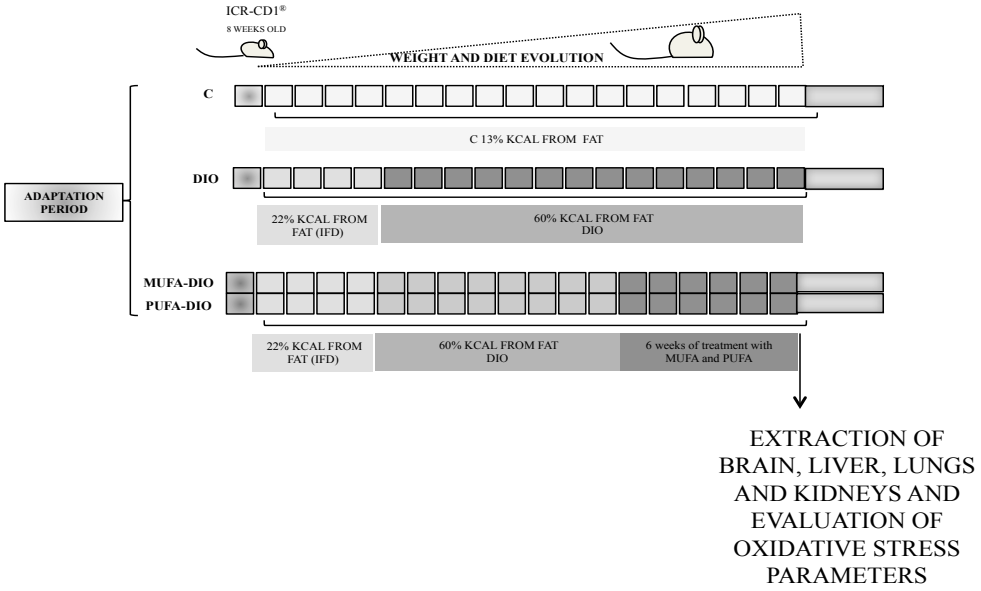
3.3.2. Effects of the dietary supplementations with 2-OHOA or with the combination of n-3 fatty acids (EPA and DHA) on the redox state of several organs from adult female diet-induced obese mice



Experimental design

At 9 weeks of age, the animals were separated into four groups (n=8, per group), similar to those in the previous design. 1) The control mice group was fed a standard maintenance diet until the end of the study. 2) The diet-induced obese mice group (DIO) was fed a high-fat diet (HFD, 60% of calories from fat, reference TD. 06414, Harlan Interfauna Iberica) for 14 weeks. 3) The MUFA-supplemented DIO mice group (MUFA-DIO) was fed a high-fat diet for 14 weeks, but during the last 6 weeks of HFD, this diet was supplemented with 2-OHOA (1500 mg of 2-OHOA per Kg of HFD, BTSA-Biotecnologías Aplicadas S.L.). 4) The n-3 PUFA-supplemented DIO mice group (PUFA-DIO) was fed a HFD for 14 weeks, this being supplemented during the last 6 weeks with the combination of EPA and DHA (3000 mg of EPA and DHA per Kg of HFD, BTSA-Biotecnologías Aplicadas S.L.). In order to progressively increase the amount of fat in the diet of groups 2, 3 and 4, they were fed with an intermediate-fat diet (IFD, 22% of calories from fat, Teklad Global 2019, Harlan Interfauna Iberica) for 4 weeks previous to the ingestion of HFD. 2-

OHOA is a synthetic derivative of oleic acid, and it is also known as 2-hydroxy-D9-cis-octadecenoic acid. The n-3 PUFA were extracted from fish (anchovy). The fatty acid supplements (2-OHOA in powdered form and n-3 PUFA in oil form) were mixed with the chow, which was of malleable consistency, and then pelleted. Animals were euthanized at 8:00 am by decapitation. The brain, liver, lungs and kidneys were frozen in liquid nitrogen and stored at -80° C until performing the oxidative stress assays.

Main results

Left and right cerebral cortex and hypothalamus

The glutathione peroxidase (GPx) and reductase (GR) activities, two antioxidant enzymes of the glutathione system, were significantly lower in the left and right cerebral cortex, respectively, of DIO mice as compared to non-DIO controls. However, DIO mice that were supplemented with MUFA or with n-3 PUFA showed significantly higher activity of these enzymes in comparison with DIO mice.

The MDA concentration was significantly higher in the hypothalamus of DIO mice with respect to non-DIO mice. In turn, the supplementation with MUFA was able to lower this concentration in comparison with non-supplemented DIO mice.

Liver

The antioxidant catalase (CAT) and GR activities were significantly lower in the liver of DIO mice as compared with that in non-DIO controls. By contrast, the GPx activity was significantly higher in DIO mice than in their respective non-DIO controls. The supplementation with MUFA or with n-3 PUFA resulted in significantly higher CAT activity in the liver in comparison with non-supplemented DIO mice. In addition, the supplementation with n-3 PUFA resulted in the lower activity of GPx and higher activity of GR in the liver of DIO mice than in non-supplemented DIO mice. No statistically significant differences were observed in the activities of GPx and GR in the liver of MUFA-supplemented DIO mice and non-supplemented DIO mice.

The activity of xanthine oxidase (XO) was significantly higher in DIO mice in comparison with that in non-DIO controls. The supplementation with MUFA or with n-3 PUFA resulted in significantly lower XO activity in the liver in comparison with non-supplemented DIO mice. In addition, the supplementation with n-3 PUFA resulted in lower GSSG/GSH ratios and higher MDA concentrations in the liver of DIO mice than in non-supplemented DIO mice.

Lungs

The activities of CAT and GR were significantly lower in the lungs of DIO mice than in non-DIO controls. However, the GPx activity was significantly higher in DIO mice than in their respective non-DIO controls. The supplementation with MUFA or with n-3 PUFA in DIO mice resulted in significantly lower activity of GPx and higher activity of GR with respect to non-supplemented DIO mice. In addition, the supplementation with n-3 PUFA in DIO mice resulted in significantly higher CAT activity than in non-supplemented DIO mice.

The XO activity, GSSG/GSH ratio and MDA concentration were significantly higher in DIO mice than in non-DIO controls. The supplementation with MUFA or with n-3 PUFA resulted in significantly lower values of these parameters in the lungs of DIO mice in comparison with non-supplemented DIO mice.

Kidneys. Renal medulla and cortex

The activities of CAT and GR were significantly lower in the renal medulla of DIO mice in comparison with non-DIO controls. By contrast, the GPx activity was significantly higher in DIO mice than in their respective non-DIO controls. The supplementation with MUFA or with n-3 PUFA resulted in significantly higher activity of CAT in the renal medulla of DIO mice in comparison with non-supplemented animals. In addition, the supplementation with MUFA resulted in significantly higher activity of GR in the renal medulla of DIO mice in comparison with non-supplemented DIO mice.

The XO activity, GSSG/GSH ratio and MDA concentration were significantly higher in DIO mice than in non-DIO controls. The supplementation with MUFA or with n-3 PUFA resulted in

significantly lower XO activity, GSSG/GSH ratio and MDA concentration in the renal medulla of DIO mice than in non-supplemented animals.

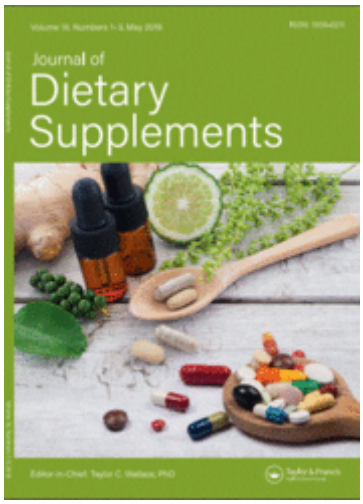
The activities of CAT and GR were significantly lower in the renal cortex of DIO mice than in non-DIO controls. However, the GPx activity was significantly higher in DIO mice than in their respective non-DIO controls. The supplementation with MUFA or with n-3 PUFA resulted in significantly higher activities of catalase and GR in the renal cortex of DIO mice in comparison with non-supplemented animals. The activity of GPx was significantly lower after the supplementation with MUFA or with n-3 PUFA in the renal cortex of DIO animals in comparison with non-supplemented DIO mice.

The XO activity, GSSG/GSH ratio and MDA concentration were significantly higher in DIO mice than in non-DIO controls. The supplementation with MUFA or with n-3 PUFA resulted in significantly lower values of these parameters in the renal cortex of DIO mice in comparison with non-supplemented DIO animals.

Partial conclusions

The supplementations with 2-OHOA or with the combination with n-3 PUFA (EPA and DHA) improve redox state in the brain, liver, lungs and kidneys, which were impaired in DIO mice, bringing them to similar values as those in the non-DIO controls.

Journal Of Dietary Supplements



SUPPLEMENTATION WITH MONOUNSATURATED AND N-3 POLYUNSATURATED FATTY ACIDS REVERTS OXIDATIVE STRESS IN VARIOUS ORGANS OF DIET-INDUCED OBESE MICE

Journal:	*Journal of Dietary Supplements*
Manuscript ID	WJDS-2019-0108
Manuscript Type:	Original Research Article
Date Submitted by the Author:	05-Jun-2019
Complete List of Authors:	Hunsche, Caroline; Universidad Complutense de Madrid Facultad de Ciencias Biologicas, Martínez de Toda, Irene; Universidad Complutense de Madrid Facultad de Ciencias Biologicas Jiménez, Beatriz ; Universidad Complutense de Madrid Facultad de Ciencias Biologicas Hernandez, Oskarina; Universidad Complutense de Madrid Facultad de Ciencias Biologicas Esperanza Díaz, Ligia; Instituto de Ciencia y Tecnología de Alimentos y Nutrición Marcos, Ascensión; Instituto de Ciencia y Tecnología de Alimentos y Nutrición De la Fuente, Mónica; Universidad Complutense de Madrid Facultad de Ciencias Biologicas
Keywords:	diet-induced obese mice, monounsaturated fatty acids, n-3 polyunsaturated fatty acids, oxidative stress, organs

SUPPLEMENTATION WITH MONOUNSATURATED AND N-3 POLYUNSATURATED FATTY ACIDS REVERTS OXIDATIVE STRESS IN VARIOUS ORGANS OF DIET-INDUCED OBESE MICE

Caroline Hunsche [1], Irene Martínez de Toda [1], Beatriz Jiménez[1], Oskarina Hernandez [1], Ligia Esperanza Díaz [2], Ascensión Marcos [2], Mónica De la Fuente[1]

[1]Department of Genetics, Physiology and Microbiology (Unit of Animal Physiology). Faculty of Biology, Complutense University of Madrid and Research Institute of Hospital 12 de Octubre (i+12).
José Antonio Novais Street, 12. 28040. Madrid. Spain.

[2]Department of Metabolism and Nutrition. Institute of Food Science, Technology and Nutrition (ICTAN). Spanish National Research Council (CSIC).
José Antonio Novais Street, 10. 28040. Madrid. Spain.

***Corresponding author:**
Mónica De la Fuente
Department of Genetics, Physiology and Microbiology (Unit of Animal Physiology). Faculty of Biology, Complutense University of Madrid
José Antonio Novais Street, 2. 28040. Madrid. Spain.
Telephone number: 0034 91 3944989
Fax number: 0034 91 3944935
Email address: mondelaf@bio.ucm.es

Running Head

Unsaturated fatty acids, redox state and DIO mice

Abstract

Obesity and its related diseases have been associated with oxidative stress. Thus, the search for nutritional strategies to ameliorate oxidative stress in obese individuals seems important. We hypothesized that the supplementations with monounsaturated and n-3 polyunsaturated fatty acids would ameliorate oxidative stress in different organs, including brain, liver, lungs, and kidneys of adult diet-induced obese (DIO) mice. Adult female ICR-CD1 mice were fed a high-fat diet (HFD) for 14 weeks. During the last 6 weeks on HFD feeding, one group of DIO mice received the same HFD, supplemented with 1500 mg of 2-hydroxyoleic acid (2-OHOA) and another group with 3000 mg of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). At the end of the experiment, several parameters of oxidative stress were assessed. The supplementations with 2-OHOA or with EPA and DHA in DIO mice were able to revert oxidative stress, enhancing the activities of catalase and glutathione reductase, as well as diminishing the activity of xanthine oxidase, the concentration of malondialdehyde and the ratio between oxidized glutathione and reduced glutathione in several organs. These reached similar values to those of control mice, which were fed a standard diet. These data suggest that the supplementations with monounsaturated and n-3 polyunsaturated fatty acids could be an effective nutritional intervention to restore an appropriate redox state in DIO mice.

Keywords

diet-induced obese mice; monounsaturated fatty acids; n-3 polyunsaturated fatty acids; oxidative stress; organs

1. Introduction

The consumption of diets high in fat and calories has been associated with the development and maintenance of obesity in both humans and rodents (Hairi and Thibault 2010; Swinburn et al. 2011). Obesity has become a global health problem and represents an important contributor to the current burden of chronic diseases (Hruby and Hu 2015).

Increasing evidence suggests that oxidative stress plays a critical role in the pathogenesis of obesity and its related diseases, such as type-2 diabetes, cardiovascular and neurodegenerative diseases (Manna and Jain 2015). It is known that moderate concentrations of reactive oxygen species (ROS) are essential to regulate biological and physiological processes, such as several signaling pathways, gene expression and apoptosis (Schieber and Chandel 2014). However, the excessive generation of these compounds (accompanied by decreased antioxidant defenses) leads to oxidative stress. Oxidative stress when maintained for a long period of time can damage cellular structures and trigger an inflammatory response, closing a detrimental feedback loop (de Heredia et al 2012; Fernández-Sánchez et al 2011). It is known that high values of xanthine oxidase (XO) activity, glutathione redox ratio (GSSG/GSH) and malondialdehyde (MDA) concentration (measured as a lipid peroxidation product) are considered markers of oxidative damage (Fernández-Sánchez et al 2011; Hunsche et al 2018; Martínez de Toda et al 2019). By contrast, inadequate endogenous antioxidant defenses, such as low values of catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GR) activities, which act as ROS scavengers, can aggravate oxidative damage (Hunsche et al 2018; Martínez de Toda et al 2019). In addition, there are other factors that also seem to contribute to oxidative stress in obesity, such as chronic inflammation (Fernández-Sánchez et al 2011), a process very related to oxidative stress (Fernández-Sánchez et al 2011), hyperglycemia (Fiorentino et al 2013), hyperleptinemia (Heymsfield et al 1999), elevated tissue lipid levels (Vincent et al 2011), adipocyte dysfunction (Trayhurn 2013), and endothelial ROS production (Egan et al 2011; Wheatcroft et al 2003). Similar to obesity, the process of aging has also been associated with oxidative stress (De la Fuente and Miquel 2009). In fact, according to the oxidative-inflammatory theory of aging, the generation of chronic oxidative stress and inflammatory stress is the basis of the age-related impairment of the functions of the organism (De la Fuente and Miquel 2009). We have found that obesity results in premature features of aging in adult mice, such as oxidative stress and immunosenescence (i.e., age-related changes of the immune system), suggesting obesity as a possible model of premature and accelerated aging (Hunsche et al 2016; 2019). Therefore, the search for strategies to ameliorate oxidative stress, and consequently to promote healthy aging in obese individuals seems important.

In this context, the consumption of diets rich in monounsaturated fatty acids (MUFAs) from olive oil and n-3 polyunsaturated fatty acids (PUFAs), namely eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), from marine-based fish oil and fish have been reported to promote beneficial effects for obesity and related diseases (including cardiovascular disease and lipid profile) (Savini et al 2013). However, the specific role of these unsaturated

fatty acids on oxidative stress remains unknown. Previous studies from our laboratory, focused on immunity, found that the supplementation with 2-hydroxyoleic acid (2-OHOA), a synthetic derivative of the MUFA oleic acid, and with EPA and DHA were able to revert oxidative stress and the altered functions in immune cells of diet-induced obese (DIO) mice (Gheorghe et al 2017; Hunsche et al 2018). We hypothesized that these supplementations would revert oxidative stress also in other non-immune organs, such as brain, liver, lungs and kidneys, of DIO mice. The aim of this study was to evaluate several oxidative stress parameters in the brain, liver, lungs and kidneys of DIO mice supplemented with 2-OHOA or with the combination of n-3 fatty acids (EPA and DHA).

2. Materials and methods

2.1. Animals

Female ICR/CD1 mice, 8 weeks of age, were purchased from Harlan Interfauna Iberica (Barcelona, Spain). During the first 5 days of acclimatization to a new environment, all mice were fed a standard maintenance diet (Teklad Global 14%). The animals were housed in polyurethane cages and maintained at a constant temperature of 22±2ºC, adequate ventilation and relative humidity of 50-60% on a 12/12h reversed light/dark cycle (lights on at 8:00 pm). The experiments procedures and handling of animals were performed with approval of the Committee for Animal Experimentation of the Complutense University of Madrid (ref. CEA-UCM 06/2012) and were conducted in accordance with the guidelines and protocols of the Royal Decree 53/2013 regarding the care and use of laboratory animals.

2.2. Experimental groups

The animals, at 9 weeks of age, were separated into four groups (n=8, per group). (1) Control mice group were fed a standard maintenance diet until the end of the study; (2) Diet-induced obese mice group (DIO) were fed a high-fat diet (HFD, 60% of calories from fat, reference TD. 06414, Harlan Interfauna Iberica) for 14 weeks; (3) MUFA-supplemented DIO mice group (MUFA-DIO) were fed a high-fat diet for 14 weeks, but received during the last 6 weeks of HFD, the supplementation with 2-OHOA (1500 mg of 2-OHOA per Kg of HFD, BTSA-Biotecnologías Aplicadas S.L.); (4) n-3 PUFA-supplemented DIO mice group (PUFA-DIO) were fed a HFD for 14 weeks, this being supplemented during the last 6 weeks with the combination of EPA and DHA (3000 mg of EPA and DHA per Kg of HFD, BTSA-Biotecnologías Aplicadas S.L.). In order to progressively increase the amount of fat in the diet of groups 2, 3 and 4, the animals were fed with an intermediate-fat diet (IFD, 22% of calories from fat, Teklad Global 2019, Harlan

Interfauna Iberica) for 4 weeks previous to the ingestion of HFD. The 2-OHOA is a synthetic derivative of oleic acid, and it is also known as 2-hydroxy-D9-cis-octadecenoic acid. The n-3 PUFA were extracted from fish (anchovy). The supplements 2-OHOA n-3 PUFA were provided in powdered form and in oil form, respectively, and were mixed into the chow, which was of malleable consistency. After the chow was uniformly mixed, it was pellet. The MUFA and n-3 PUFA supplementation doses were chosen according to a previous study, in which the same supplemented doses were associated with changes in the gut microbiota of DIO mice (Mujico et al 2013). The animals and the experimental design used in this study were the same as those used in a previously published paper that studied the same supplementations with monounsaturated and n-3 polyunsaturated fatty acids on the immune functions and oxidative stress of spleen leukocytes (Gheorghe et al 2017). During the entire study, all mice were given *ad libitum* access to water and diet. The full compositions of all the diets used are shown in Table 1. Weight and food intake were measured every week throughout the study (data previously published) (Gheorghe et al 2017).

2.3 Collection of organs

Animals were euthanized at 8:00 am by decapitation, and no anesthetic was used to avoid the effect of anesthesia on the results. Organs were extracted *post-mortem* immediately and washed carefully with phosphate buffer, pH 7.4. The brain, liver, lungs and kidneys were frozen in liquid nitrogen and stored at -80° C until performing the oxidative stress assays.

2.4. Catalase activity assay

The activity of catalase (CAT) was determined following the method previously described (Alvarado et al 2006). The assays were performed using aliquots of the homogenized tissue samples (50 mg/ml, for kidneys and lungs, and 25 mg/ml, for liver) in phosphate buffer (66 mM, pH 8.4) and centrifuged at 3200 *g* for 20 min at 4°C. The supernatant extract (30 µl) was mixed with 670 µl of hydrogen peroxide (H_2O_2) (14 mM of H_2O_2 in phosphate buffer) (Merck, Germany). The enzymatic assay was followed using spectrophotometry for 80 seconds at 240 nm through the decomposition of H_2O_2 into $H_2O + O_2$. Protein content of the samples was assessed following the bicinchoninic acid (BCA) protein assay kit protocol (Sigma-Aldrich, Madrid, Spain). The results were expressed as units (U) of enzymatic activity per milligram of protein (U CAT/mg protein).

2.5. Glutathione peroxidase activity assay

The glutathione peroxidase (Gpx) activity was determined according to the method previously described (Alvarado et al 2006). The assays were performed with aliquots of the homogenate tissue samples (50 mg/ml, for brain, kidneys and lungs, and 25 mg/ml for liver) in phosphate buffer (50 mM, pH 7.4) and centrifuged at 3200 *g* for 20 min at 4°C. The total activity was determined using cumene hydroperoxide (Sigma-Aldrich), which carried out the oxidation of β-nicotinamide adenine dinucleotide phosphate, in its reduced form (β-NADPH, Sigma-Aldrich, Spain), in the presence of glutathione reductase (Sigma-Aldrich). The reaction was measured spectrophotometrically by the decrease of the absorbance at 340 nm. The protein contents were evaluated following the previously mentioned protocol. The results were expressed as miliunits of enzymatic activity per milligram of proteins (mU GPx/mg protein).

2.6. Glutathione reductase activity assay

The glutathione reductase (GR) activity was measured by the technique previously described (Alvarado et al 2006). The assays were performed with aliquots of the homogenate tissue samples (50 mg/ml, for kidneys and lungs, and 25 mg/ml for liver) in phosphate buffer 50 mM, pH 7.4 with 6.3 mM ethylenediaminetetraacetic acid (EDTA), and centrifuged at 3200 *g* for 20 min at 4°C. The total activity was measured through the oxidation of NADPH spectrophotometrically at 340 nm. The protein contents of samples were evaluated following the previously described protocol. The results were expressed as milliunits of enzymatic activity per milligram of proteins (mU GR/mg protein).

2.7. Glutathione concentrations assay

Both reduced (GSH) and oxidized (GSSG) concentrations of glutathione were measured using a fluorometric method. This method is based on the reaction of a fluorescence probe, o-phthaldialdehyde (OPT; Sigma-Aldrich), with GSH at pH 8 and with GSSG at pH 12, which generates a fluorescence derivative. The tissue samples were homogenized (50 mg/ml, for kidneys and lungs, and 25 mg/ml for liver) in sodium phosphate-EDTA buffer (0.1 M, pH 8) and proteins were precipitated by adding 5 µl of 60% perchloric acid ($HClO_4$) (60%, Sigma-Aldrich). The homogenate tissue samples were centrifuged at 9500 *g* for 10 min at 4°C and supernatants were maintained in ice for the measurement of GSH and GSSG concentrations. For GSH content determination, 10 µl of the supernatant, 190 µl of phosphate-EDTA buffer and 20 µl of OPT solution (1 mg/ml in methanol) were added to a 96-well black plate (Nunc, Roskilde, Denmark), and incubated at room temperature for 15 minutes. Fluorescence was determined in a plate reader (Fluostar Optima, BMG Labtech, Spain) using excitation at 350 nm and emission detection at 420 nm. For the measurement of GSSG contents, 8 µl of N-ethylmaleimide (NEM, 0,04M, Sigma-Aldrich) were added to each well and incubated at room temperature for 30 minutes. Then, 182 µl of sodium hydroxide (NaOH) (0.1 N, Panreac Quimica SA,

Barcelona, Spain) with 20 µl of OPT solution were added to a 96-well black plate. After incubation (room temperature, 15 min), fluorescence was measured as previously described for GSH determination. Protein concentration of the samples was measured following the bicinchroninic acid protein assay kit protocol (Sigma-Aldrich). The results were analyzed with GSH and GSSG standard curves at different concentrations and expressed as nmol/mg protein. The GSSG/GSH ratios were then calculated for each sample.

2.8. Xanthine oxidase (XO) activity assay

Xanthine oxidase (XO) activity was measured, by a fluorescence assay, in homogenates of tissues, using a commercial kit (Amplex Red Xanthine/Xanthine Oxidase Assay Kit, Molecular Probes, Paisley, UK). The hydrogen peroxide (H_2O_2) produced by XO reacts with horseradish peroxidase (HRP) present in the reaction mixture and generates a fluorescent oxidation compound resorufin whose fluorescence is measured in a plate reader (Fluorestar Optima, BMG Labtech Biomedal, Spain). Tissue samples were homogenized (50 mg/ml, for kidneys and lungs, and 25 mg/ml for liver) in phosphate buffer (50 mM, pH 7.4) containing 1 mM EDTA. The homogenate was centrifuged (5000 *g*), and the supernatant (50 µl) was collected and incubated with 50 µl working solution of Amplex Red reagent (100 µM) containing HRP (0.4 U/ml) and xanthine (200 µM). After 30 min of incubation at 37°C, measurement of fluorescence was performed in the microplater reader, using excitation at 530 nm and emission detection at 595 nm. XO supplied in the kit was used as the standard. Protein content of the samples was assessed using the BCA protein assay (Sigma-Aldrich). The results were expressed as milliunits of enzymatic activity per milligram of protein (mU XO/mg protein).

2.9. Malondialdehyde (MDA) assay

The estimation of malondialdehyde (MDA), a marker of lipid peroxidation, was evaluated using a colorimetric assay kit (BioVision, Mountain View, CA, USA), which measures the reaction of MDA with thiobarbituric acid (TBA) and the MDA-TBA adduct formation. The tissue samples were homogenized (50 mg/ml, for brain, kidneys and lungs, and 25 mg/ml for liver) in 300 µl of MDA lysis buffer with 3 µl butylhydroxytoluene (BHT) (X100) and then centrifuged (13000 *g*, 10 min, 4°C) to remove insoluble material. An aliquot (200 µl) of each supernatant was added to 600 µl of thiobarbituric acid (TBA) and incubated at 95°C for 60 min. The samples were then maintained in an ice bath for 10 min and 200 µl from each 800 µl reaction mixture were placed into a 96-well microplate for spectrophotometric measurement at 532 nm. Protein concentration was measured following the BCA protein assay kit (Sigma-Aldrich). The results were obtained using a MDA standard curve at different concentrations and expressed as nmol MDA/mg protein.

2.10. Statistical analysis

The statistical analysis of results was performed in SPSS IBM, version 25.0 (SPSS, Inc., Chicago, USA). The data were expressed as mean ± standard error of the mean (SEM). Each value is the mean of the data from an assay performed in duplicate or triplicate. The normality of the samples was checked by the Kolmogorov-Smirnov test and homogeneity of variances with the Levene test. The data were statistically evaluated by one-way ANOVA followed by Tukey´s post hoc test for homogenous variances. Games-Howell´s post hoc test was used for unequal variances. $P<0.05$ was considered statistically significant.

3. Results

3.1. Left and right cerebral cortex and hypothalamus

The glutathione peroxidase (GPx) and reductase (GR) activities, two antioxidant enzymes of the glutathione system, were significantly lower in the left ($P<0.001$; Fig. 1A) and right cerebral cortex ($P=0.026$; Fig. 1B), respectively, of DIO mice as compared to non-DIO controls. However, DIO mice that were supplemented with MUFA or with n-3 PUFA showed significantly higher activity of these enzymes in comparison with DIO mice ($P=0.03$ and $P<0.001$ for GPx activity, respectively; Fig. 1A) and ($P=0.04$ and $P=0.01$ for GR activity, respectively; Fig. 1B).

The MDA concentrations, which are an indicator of lipid oxidation and oxidative damage in cells, was significantly higher in the hypothalamus of DIO mice with respect to non-DIO mice ($P=0.04$; Fig. 1C). In turn, the supplementation with MUFA was able to lower the concentrations of MDA in comparison with non-supplemented DIO mice ($P=0.009$; Fig. 1C). No significant differences were observed between PUFA-DIO supplemented and non-supplemented mice regarding the concentrations of MDA in the hypothalamus ($P=0.4$; Fig 1C).

3.2. Liver

The antioxidant catalase and glutathione reductase activities were significantly lower in the liver of DIO mice as compared with that in non-DIO controls ($P=0.008$ and $P=0.012$, respectively; Fig. 2A and 2C). By contrast, the GPx activity was significantly higher in DIO mice than in their respective non-DIO controls ($P=0.015$; Fig. 2B). The supplementation with MUFA or with n-3 PUFA resulted in significantly higher CAT activity in the liver ($P=0.015$ and $P<0.001$, respectively; Fig. 2A) in comparison with non-supplemented DIO mice. In addition, the supplementation with

n-3 PUFA resulted in lower activity of GPx and higher activity of GR in the liver of DIO mice (P<0.001 and P=0.038, respectively; Fig. 2B and 2C) than in non-supplemented DIO mice. No statistically significant differences were observed in the activities of GPx and GR in the liver of MUFA-supplemented DIO mice and non-supplemented DIO mice (P=0.073 and P=0.108, respectively; Fig. 2B and 2C).

The activity of xanthine oxidase (XO), which is associated with the production of free radicals, as well as the GSSG/GSH ratio, an indicator of oxidative stress, were significantly higher in DIO mice in comparison with those in non-DIO controls (P=0.001 and P<0.001, respectively; Fig. 2D and 2E). No statistically significant differences were observed in the concentrations of MDA in the liver of DIO mice and non-DIO mice (P=0.564; Fig. 2F). The supplementation with MUFA or with n-3 PUFA resulted in significantly lower XO activity in the liver (P=0.028 and P=0.032, respectively; Fig. 2D) in comparison with non-supplemented DIO mice. In addition, the supplementation with n-3 PUFA resulted in lower GSSG/GSH ratios and higher MDA concentrations in the liver of DIO mice (P=0.017 and P=0.006, respectively; Fig. 2E and 2F) than in non-supplemented DIO mice. No statistically significant differences were observed in the GSSG/GSH ratios and MDA concentrations in the liver of MUFA-supplemented DIO mice and non-supplemented DIO mice (P=0.510 and P=0.079, respectively; Fig. 2E and 2F).

3.3. Lungs

The activities of CAT and GR were significantly lower in the lungs of DIO mice that in non-DIO controls (P<0.001and P<0.001, respectively; Fig. 3A, 3C). However, the GPx activity was significantly higher in DIO mice than in their respective non-DIO controls (P<0.001; Fig. 3B). The supplementation with MUFA or with n-3 PUFA in DIO mice resulted in significantly lower activity of GPx (P<0.001 and P<0.001, respectively; Fig. 3B) and higher activity of GR (P=0.001 and P=0.001, respectively; Fig. 3C) with respect to non-supplemented DIO mice. In addition, the supplementation with n-3 PUFA in DIO mice resulted in significantly higher CAT activity (P<0.001; Fig. 3A) than in non-supplemented DIO mice. No statistically significant differences were observed in the CAT activity in the lungs of MUFA-supplemented DIO mice and non-supplemented DIO mice (P=0.062; Fig. 3A).

The XO activity, GSSG/GSH ratios and MDA concentrations were significantly higher in DIO mice than in non-DIO controls (P<0.001, P<0.001 and P<0.001, respectively; Fig. 3D, 3E and 3F). The supplementation with MUFA or with n-3 PUFA resulted in significantly lower XO activity (P=0.005 and P<0.001, respectively; Fig. 3D), GSSG/GSH ratios (P=0.002 and P<0.001, respectively; Fig. 3E) and MDA concentrations (P<0.001 and P<0.001, respectively; Fig. 3F) in the lungs of DIO mice in comparison with non-supplemented DIO mice.

3.4. Kidneys: Renal medulla and cortex

The activities of CAT and GR were significantly lower in the renal medulla of DIO mice in comparison with non-DIO controls (P=0.001 and P=0.006, respectively; Fig. 4A, 4C). By contrast, the GPx activity was significantly higher in DIO mice than in their respective non-DIO controls (P=0.025; Fig. 4B). The supplementation with MUFA or with n-3 PUFA resulted in significantly higher activity of catalase (P=0.001 and P<0.001, respectively; Fig. 4A) and lower activity of GPx (P=0.024 and P=0.015, respectively; Fig. 4B) in the renal medulla of DIO mice in comparison with non-supplemented animals. In addition, the supplementation with MUFA resulted in significantly higher GR activity (P=0.022; Fig. 4C) in the renal medulla of DIO mice in comparison with non-supplemented DIO mice. No statistically significant differences were observed in the GR activity in the renal medulla of n-3 PUFA-supplemented DIO mice and non-supplemented DIO mice (P=0.995; Fig. 4C).

The XO activity, GSSG/GSH ratios and MDA concentrations were significantly higher in DIO mice than in non-DIO controls (P=0.032, P<0.001 and P=0.034, respectively; Fig. 4D, 4E and 4F). The supplementation with MUFA or with n-3 PUFA resulted in significantly lower XO activity (P=0.003 and P=0.003, respectively; Fig. 4D), GSSG/GSH ratios (P=0.015 and P<0.001, respectively; Fig. 4E) and MDA concentrations (P=0.001 and P<0.001, respectively; Fig. 4F) in the renal medulla of DIO mice than in non-supplemented animals.

The activities of CAT and GR were significantly lower in the renal cortex of DIO mice than in non-DIO controls (P=0.020 and P<0.001, respectively; Fig. 5A, 5C). However, the GPx activity was significantly higher in DIO mice than in their respective non-DIO controls (P<0.001; Fig. 5B). The supplementation with MUFA or with n-3 PUFA resulted in significantly higher activities of catalase (P=0.039 and P=0.011, respectively; Fig. 5A) and GR (P=0.003 and P=0.007, respectively; Fig. 5C) in the renal cortex of DIO mice in comparison with non-supplemented animals. The activity of GPx was significantly lower after the supplementation with MUFA or with n-3 PUFA (P=0.006 and P=0.039, respectively; Fig. 5B) in the renal cortex of DIO animals in comparison with non-supplemented DIO mice.

The XO activity, GSSG/GSH ratios and MDA concentrations were significantly higher in DIO mice than in non-DIO controls (P<0.001, P=0.001 and P=0.002, respectively; Fig. 5D, 5E and 5F). The supplementation with MUFA or with n-3 PUFA resulted in significantly lower XO activity (P<0.001 and P=0.001, respectively; Fig. 5D), GSSG/GSH ratios (P=0.013 and P=0.001, respectively; Fig. 5E) and MDA concentrations (P<0.001 and P=0.002, respectively; Fig. 5F) in the renal cortex of DIO mice in comparison with non-supplemented DIO animals.

4. Discussion

Although we previously demonstrated that the supplementations with monounsaturated (2-OHOA) and n-3 polyunsaturated (DHA plus EPA) fatty acids improved the functions and redox state of immune cells (Gheorghe et al

2017; Hunsche et al 2018), the effect of these supplementations on the oxidative stress of the brain, liver, lungs and kidneys from diet-induced obese (DIO) mice, have not been studied.

As shown in our previously published paper, which used the same animals, the mice fed a high-fat diet gained significantly more body weight than controls (fed a standard diet) (Gheorghe et al 2017). In addition, other studies from our laboratory confirmed that the high-fat diet intake is a good model to induce obesity in ICR-CD1 mice. These animals also showed common features of obesity, such as elevated levels of triglycerides, total cholesterol and systolic arterial pressure (Hunsche et al 2016; 2019; 2018). In turn, we observed that the supplementation with 2-OHOA, but not with n-3 PUFA, resulted in significantly lower body weight of high-fat fed mice than that of non-supplemented high-fat fed animals (Gheorghe et al 2017). In this sense, 2-OHOA seems to promote body weight loss through the induction of UCP-1 (uncoupling protein-1) expression in the adipose tissue, a process probably accompanied by enhanced energy expenditure (Vögler et al 2008). Regarding the non-effects of the supplementation with n-3 PUFA on body weight, a previous study also found that this supplementation did not reduce body weight of obese individuals (Du et al 2015).

In addition, our current results showed increased oxidative stress in the brain (left and right cerebral cortex and hypothalamus), liver, lungs and kidneys (renal medulla and cortex) of diet-induced obese (DIO) mice in comparison with non-DIO controls. Thus, these DIO animals showed lower antioxidant defenses, such as CAT and GR activities, as well as higher production of oxidants, including XO activity, GSSG/GSH ratio and lipid peroxidation (MDA concentration) than non-DIO mice. The increased oxidative stress seems to be associated with lipotoxicity in these organs, which is generated when triglycerides are inappropriately stored in non-adipose tissues. The excessive accumulation of intracellular triglycerides is known to reduce the efficacy of the electron transport chain, causing the release of ROS and the generation of oxidative damage (Savini et al 2013). In agreement with our current findings, previous reports also indicate that obesity increased oxidative stress in a variety of organs, such as cerebral cortex (Freeman and Keller 2012), liver and kidneys (Noeman et al 2011; Yuzefovych et al 2013). These studies suggest that oxidative stress is related to adiposity, lipotoxicity, mitochondrial dysfunction and endoplasmic reticulum stress in a variety of organs from obese rodents (Freeman and Keller 2012; Noeman et al 2011; Yuzefovych et al 2013). Moreover, another study showed that high-fat diet feeding increased mitochondrial hydrogen peroxide (H_2O_2) production, causing abnormal expression of antioxidant enzymes (such as CAT) (Rindler et al 2013). Interestingly, we found higher values of glutathione peroxidase (GPx) activity (an antioxidant enzyme) in the liver, lungs and kidneys of DIO mice in comparison with non-DIO mice. However, lower values of GPx activity were observed in the brain (right cerebral cortex) of DIO mice in comparison with non-DIO mice. The higher activity of this antioxidant enzyme could possibly be explained as a compensatory mechanism to protect these cells against oxidative damage (Vincent et al 2011). Similarly, another study from our laboratory also found increased values of GPx in peritoneal leukocytes of DIO mice (Hunsche et al 2019). Thus, the activity of this enzyme has been reported to increase or decrease in response to

oxidative damage depending on the moment of its evolution and the amount of peroxides generated (Liu et al 2002; Yan and Harding 1997).

In turn, the dietary supplementations with 2-OHOA or with the combination of EPA and DHA in general were able to restore oxidative stress in the brain (with higher values of GPx and GR activities and lower values of MDA concentration) as well as in the liver, lungs and kidneys of DIO mice (with higher values of CAT and GR activities and lower values of XO activity, GSSG/GSH ratio and MDA concentration) in comparison with non-supplemented DIO mice. In all cases, the values showed similar levels to those of non-DIO controls. In agreement, our previous studies in spleen and peritoneal immune cells also showed amelioration of oxidative stress after the supplementations with 2-OHOA or n-3 PUFA (EPA and DHA) (with increased values of catalase activity and total glutathione concentration and decreased values of XO activity, GSSG/GSH ratio and MDA concentration) (Gheorghe et al 2017; Hunsche et al 2018). Furthermore, another study demonstrated that oleic acid exhibited an antioxidant activity in *Caenorhabditis elegans*, which was regulated by the forkhead transcription factor DAF-16/FOXO (forkhead box protein O) (Wei et al 2016). Other studies also showed the antioxidant properties of n-3 PUFA against lead acetate-induced toxicity in the liver and kidneys of rats (Abdou and Hassan 2014), as well as in the brain of rats exposed to ethanol during gestation (Patten et al 2013). One of the possible mechanisms involved in these effects could be the incorporation of unsaturated fatty acids into the cell membranes, which occurs in all tissues of the body, following their consumption. In addition, these fatty acids are known to modulate cell functions, by modifying membrane fluidity, lipid peroxide formation, eicosanoid production and gene regulation (De Pablo and Alvarez de Cienfuegos 2000; Surette 2008).

Interestingly, the supplementation with 2-OHOA was not able to improve the activity of GR and the GSSG/GSH ratio in the liver of DIO mice. In addition, the supplementation with n-3 PUFA (EPA and DHA) increased the lipid peroxidation in the liver of DIO mice compared to non-supplemented DIO mice. These findings showed that both supplementations, especially with n-3 PUFA, were less effective in restoring an appropriate redox state in the liver of DIO mice in comparison with the other organs of DIO animals. This could possibly be explained by the central role of the liver in the metabolism of fatty acids and by fact that PUFA supplemented DIO mice received a higher dose (3000 mg of EPA and DHA per Kg of HFD) than that of MUFA supplemented DIO mice (1500 mg of 2-OHOA per Kg of HFD). Thus, it is possible that the lipototoxity and oxidative stress generated in the liver as a result of obesity could not be completely reversed by these supplementations. Similarly, our previous findings also showed higher susceptibility to lipid peroxidation in the spleen and peritoneal leukocytes of n-3 PUFA supplemented DIO mice than MUFA supplemented DIO mice (Gheorghe et al 2017; Hunsche et al 2018).

In conclusion, diet-induced obesity resulted in increased oxidative stress in the brain, liver, lungs and kidneys of mice. In turn, both supplementations with 2-OHOA and n-3 PUFA (EPA and DHA) were able to restore the redox state in these organs, bringing the values to similar levels to those of non-DIO controls. Thus, these supplementations

with 2-OHOA and n-3 PUFA could be a promising nutritional strategy to reduce the progression of obesity-related diseases as well as to promote a healthy aging, probably through the reduction of oxidative stress.

Declaration of interest

The authors declare no conflict of interest.

Funding

This work was supported by grants from Ministry of Science and Innovation (BFU2011-30336), the Research Group of Madrid Complutense University (910379ENEROINN), FIS (PI15/01787) from the Carlos III Health Institute of the European Union (ISCIII-FEDER), the PRONAOS study (CDTI 20081114) and BTSA-Applied Biotechnologies S.L.

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FIGURE LEGENDS

Figure 1. Oxidative stress parameters in the brain. **(A)** Glutathione peroxidase (GPx) activity in the left cerebral cortex (mU/mg protein). **(B)** Glutathione reductase (GR) activity in the right cerebral cortex (mU/mg protein). **(C)** Malondialdehyde (MDA) concentrations in the hypothalamus (nmol/mg protein). Each column represents the mean ± *SEM* of 8 values corresponding to that number of animals and each value being the mean of duplicate or triplicate assays. *** $P<0.001$; * $P<0.05$ with respect to the values in non-obese control mice. ### $P<0.001$; ## $P<0.01$; # $P<0.05$ with respect to the values in DIO mice.

Figure 2. Oxidative stress parameters in the liver. **(A)** Catalase (CAT) activity (U/mg protein). **(B)** Glutathione peroxidase (GPx) activity (mU/mg protein). **(C)** Glutathione reductase (GR) activity (mU/mg protein). **(D)** Xanthine oxidase (XO) activity (mU/mg protein). **(E)** oxidized glutathione (GSSG)/ reduced glutathione (GSH) ratio. **(F)** Malondialdehyde (MDA) concentrations. Each column represents the mean ± *SEM* of 8 values corresponding to that number of animals and each value being the mean of duplicate or triplicate assays. *** $P<0.001$; ** $P<0.01$; *$P<0.05$ with respect to the values in non-obese control mice. ### $P<0.001$; ## $P<0.01$; # $P<0.05$ with respect to the values in DIO mice.

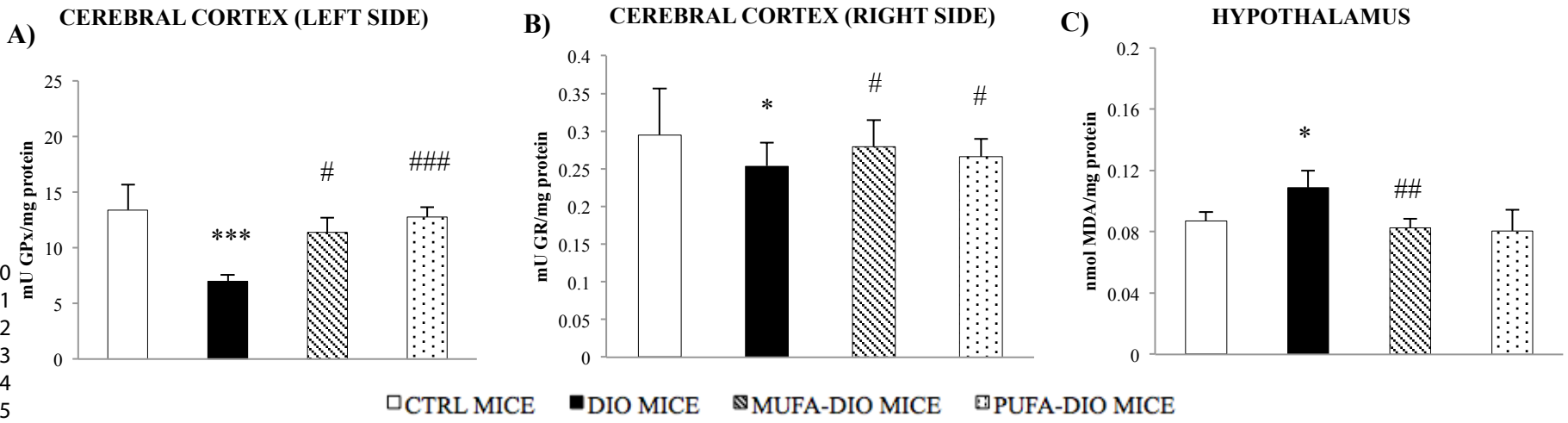
Figure 3. Oxidative stress parameters in the lungs. **(A)** Catalase (CAT) activity (U/mg protein). **(B)** Glutathione peroxidase (GPx) activity (mU/mg protein). **(C)** Glutathione reductase (GR) activity (mU/mg protein). **(D)** Xanthine oxidase (XO) activity (mU/mg protein). **(E)** oxidized glutathione (GSSG)/ reduced glutathione (GSH) ratio. **(F)** Malondialdehyde (MDA) concentrations. Each column represents the mean ± *SEM* of 8 values corresponding to that number of animals and each value being the mean of duplicate or triplicate assays. *** $P<0.001$ with respect to the values in non-obese control mice. ### $P<0.001$; ## $P<0.01$ with respect to the values in DIO mice.

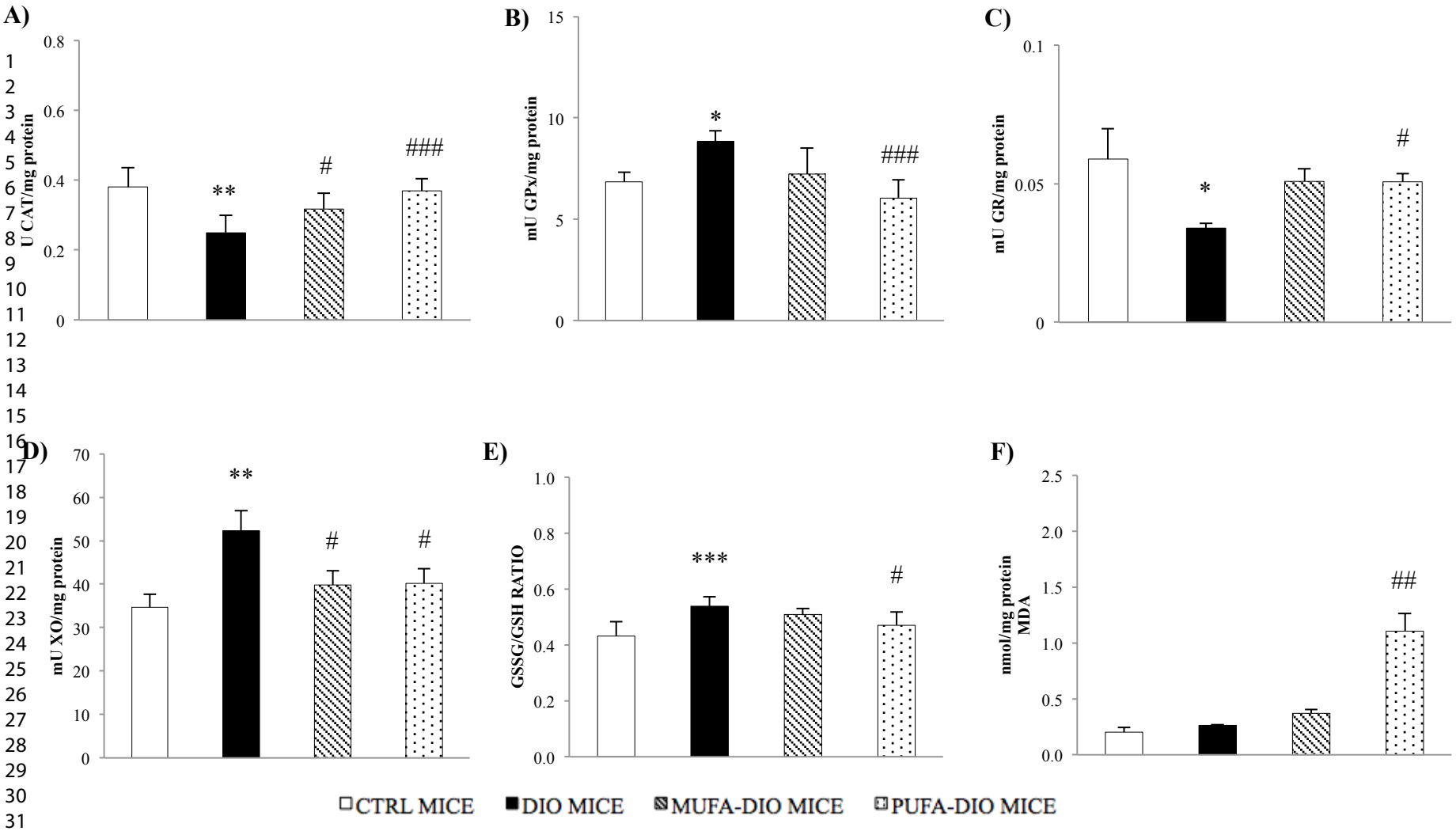
Figure 4. Oxidative stress parameters in the renal medulla. **(A)** Catalase (CAT) activity (U/mg protein). **(B)** Glutathione peroxidase (GPx) activity (mU/mg protein). **(C)** Glutathione reductase (GR) activity (mU/mg protein). **(D)** Xanthine oxidase (XO) activity (mU/mg protein). **(E)** Oxidized glutathione (GSSG)/ reduced glutathione (GSH) ratio. **(F)** Malondialdehyde (MDA) concentrations. Each column represents the mean ± *SEM* of 8 values corresponding to that number of animals and each value being the mean of duplicate or triplicate assays. *** $P<0.001$; ** $P<0.01$; *$P<0.05$ with respect to the values in non-obese control mice. ### $P<0.001$; ## $P<0.01$; # $P<0.05$ with respect to the values in DIO mice.

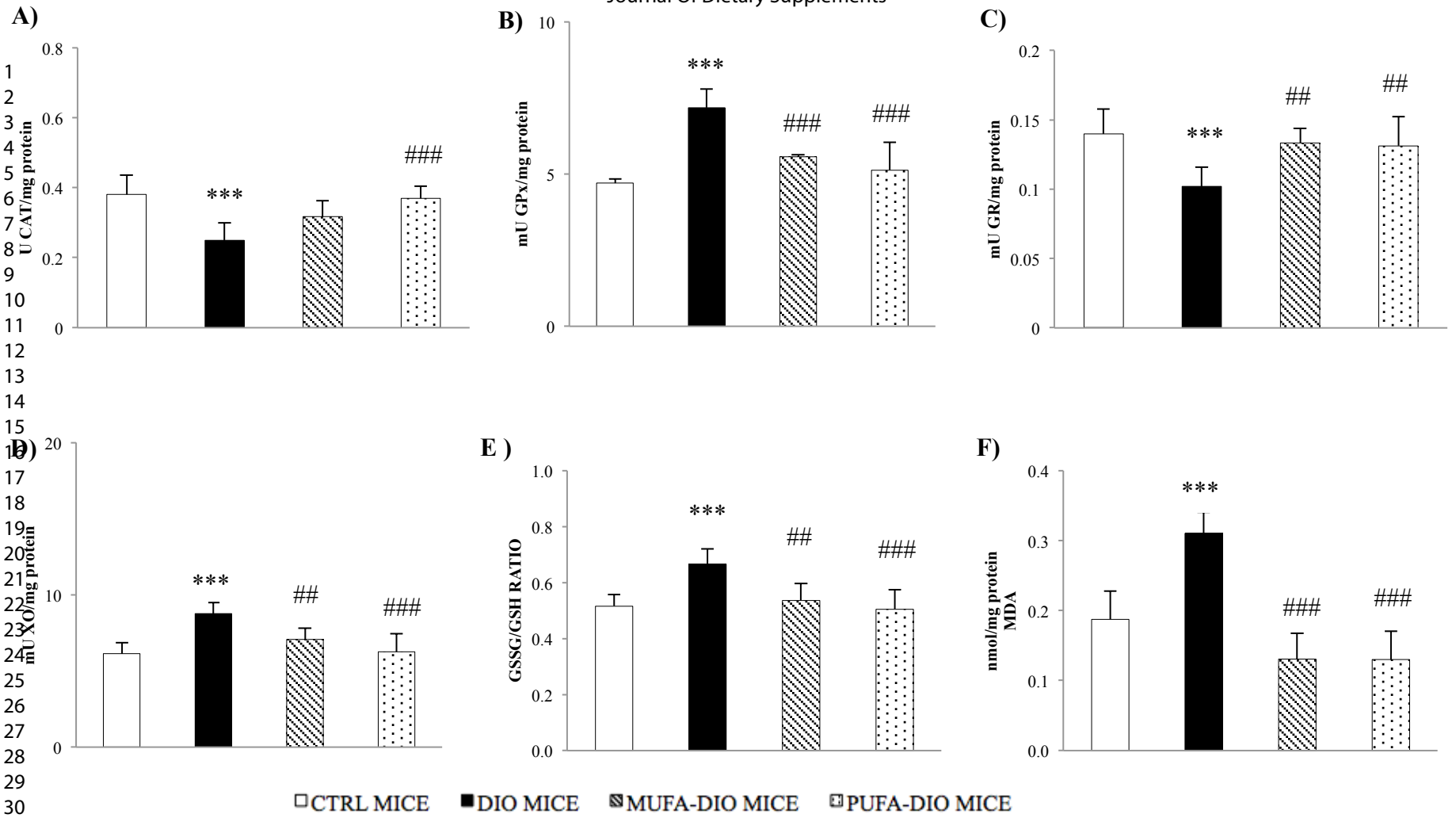
Figure 5. Oxidative stress parameters in the renal cortex. **(A)** Catalase (CAT) activity (U/mg protein). **(B)** Glutathione peroxidase (GPx) activity (mU/mg protein). **(C)** Glutathione reductase (GR) activity (mU/mg protein). **(D)** Xanthine oxidase (XO) activity (mU/mg protein). **(E)** Oxidized glutathione (GSSG)/ reduced glutathione (GSH) ratio. **(F)** Malondialdehyde (MDA) concentrations. Each column represents the mean ± *SEM* of 8 values corresponding to that number of animals and each value being the mean of duplicate or triplicate assays. *** $P<0.001$; ** $P<0.01$; *$P<0.05$ with respect to the values in non-obese control mice. ### $P<0.001$; ## $P<0.01$; # $P<0.05$ with respect to the values in DIO mice.

Table 1. Composition of diets

Components	Standard maintenance diet	Intermediate-fat diet	High-fat diet
Energy (Kcal/g)	2.9	3.3	5.1
Protein (%)	14.3	19.0	23.5
Carbohydrate (%)	48.0	44.9	27.3
Fat (%)	4.0	9.0	34.3
Saturated (%)	0.6	1.2	12.5
Monounsaturated (%)	0.7	1.7	16.1
Polyunsaturated (%)	2.1	4.4	5.4
Protein (% of energy)	20.0	23.0	18.4
Carbohydrate (% of energy)	67.0	55.0	21.3
Fat (% of energy)	13.0	22.0	60.3
Fatty acid composition (%)			
C16:0 Palmitic	0.5	0.9	8.2
C18:0 Stearic	0.1	0.2	3.9
C18:1 Oleic	0.7	1.7	14.7
C18:2 Linoleic	2.0	3.9	4.7
C18:3 Linolenic	0.1	0.4	0.5


A)
CEREBRAL CORTEX (LEFT SIDE)
mU GPx/mg protein
25
20
15
10
5
0
***
#
###
B)
CEREBRAL CORTEX (RIGHT SIDE)
mU GR/mg protein
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
*
#
#
C)
HYPOTHALAMUS
nmol MDA/mg protein
0.2
0.16
0.12
0.08
0.04
0
*
##
CTRL MICE
DIO MICE
MUFA-DIO MICE
PUFA-DIO MICE


A)
U CAT/mg protein
0.8
0.6
0.4
0.2
0
**
#
###
B)
mU GPx/mg protein
15
10
5
0
*
###
C)
mU GR/mg protein
0.1
0.05
0
*
#
D)
mU XO/mg protein
70
60
50
40
30
20
10
0
**
#
#
E)
GSSG/GSH RATIO
1.0
0.8
0.6
0.4
0.2
0.0
***
#
F)
nmol/mg protein MDA
2.5
2.0
1.5
1.0
0.5
0.0
##
CTRL MICE
DIO MICE
MUFA-DIO MICE
PUFA-DIO MICE


A)
U CAT/mg protein
0.8
0.6
0.4
0.2
0
***
###
B)
mU GPx/mg protein
10
5
0
***
###
###
C)
mU GR/mg protein
0.2
0.15
0.1
0.05
0
***
##
##
D)
mU XO/mg protein
20
10
0
***
##
###
E)
GSSG/GSH RATIO
1.0
0.8
0.6
0.4
0.2
0.0
***
##
###
F)
nmol/mg protein MDA
0.4
0.3
0.2
0.1
0.0
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CTRL MICE
DIO MICE
MUFA-DIO MICE
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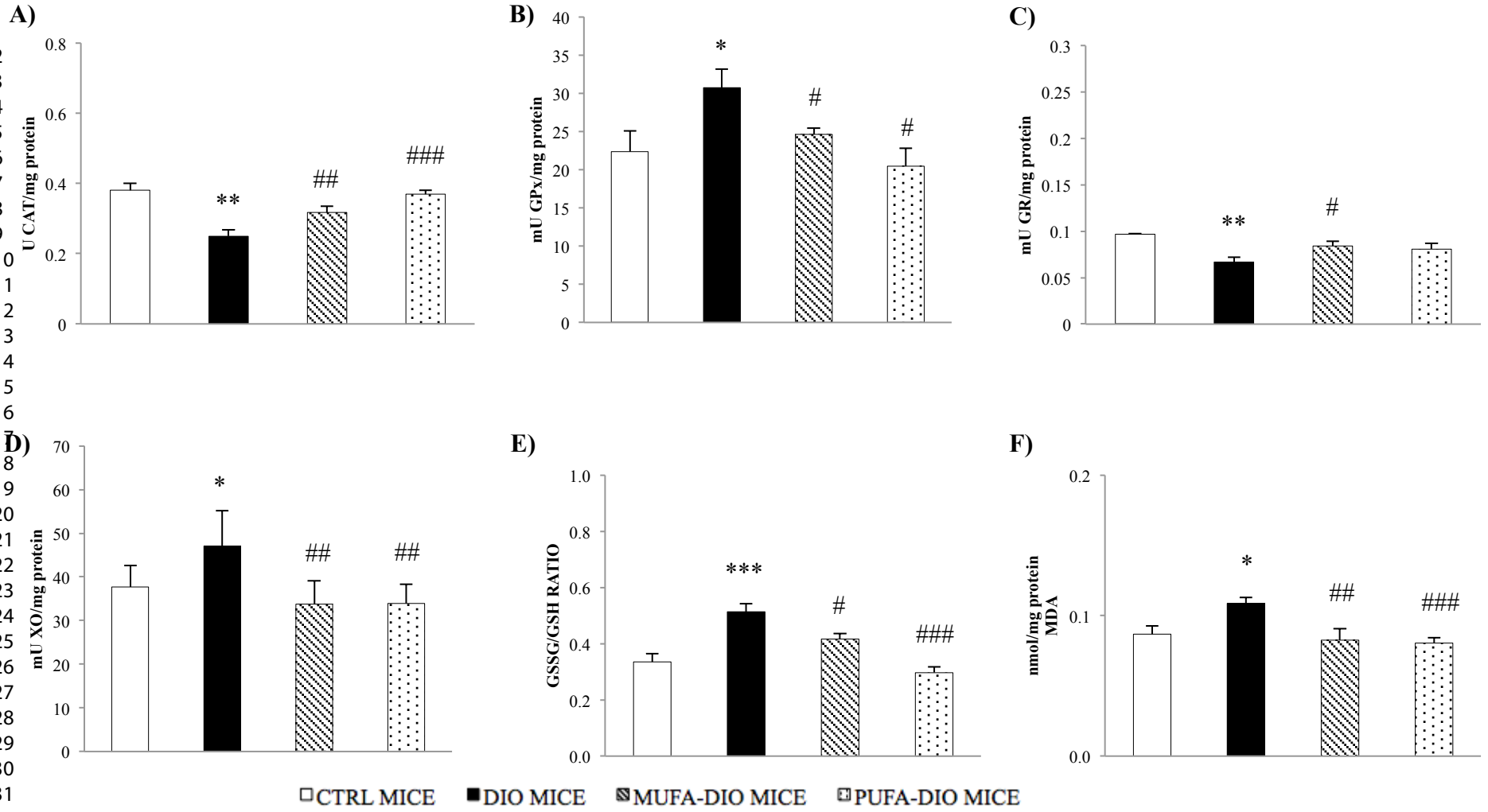
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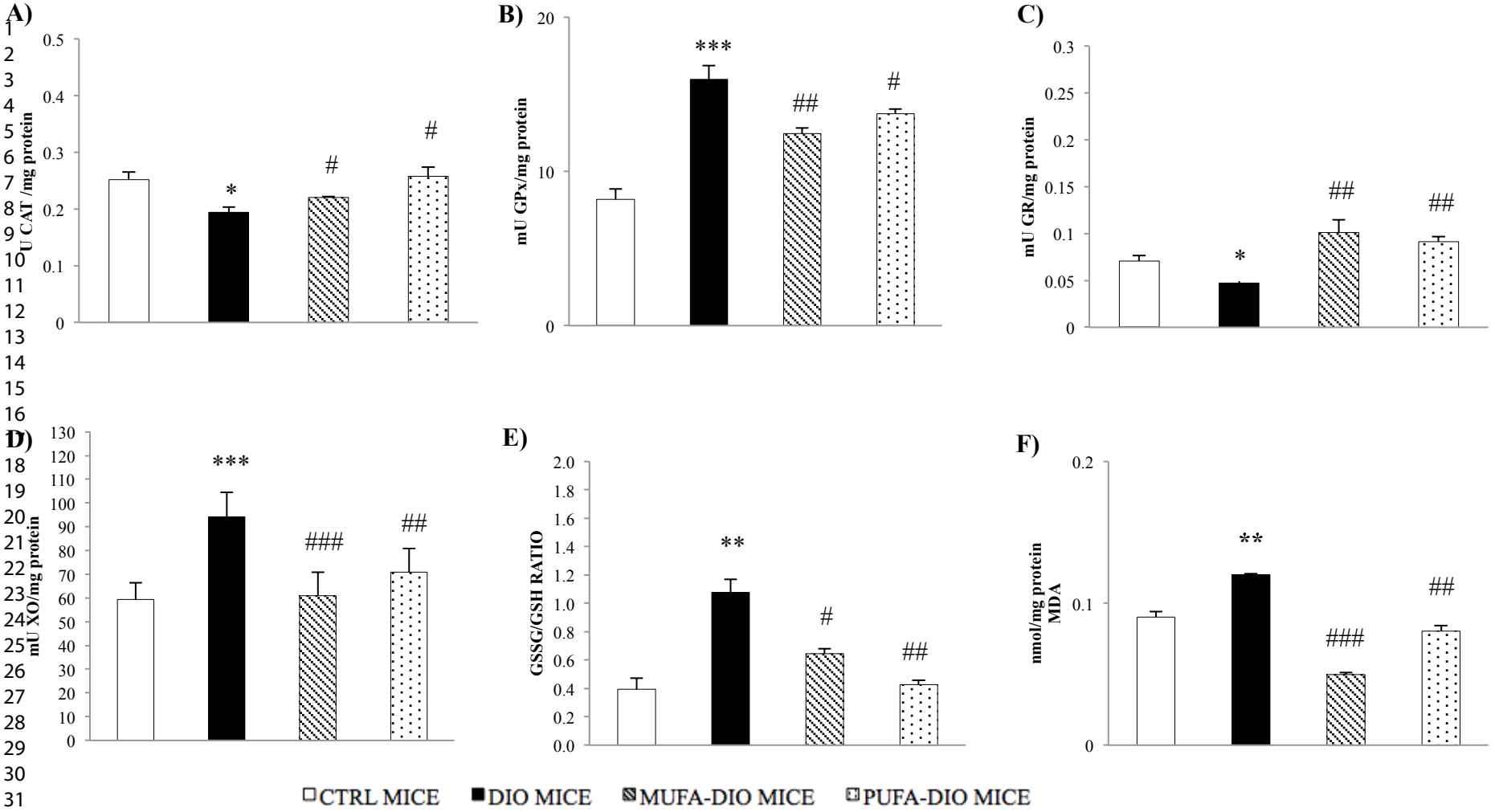
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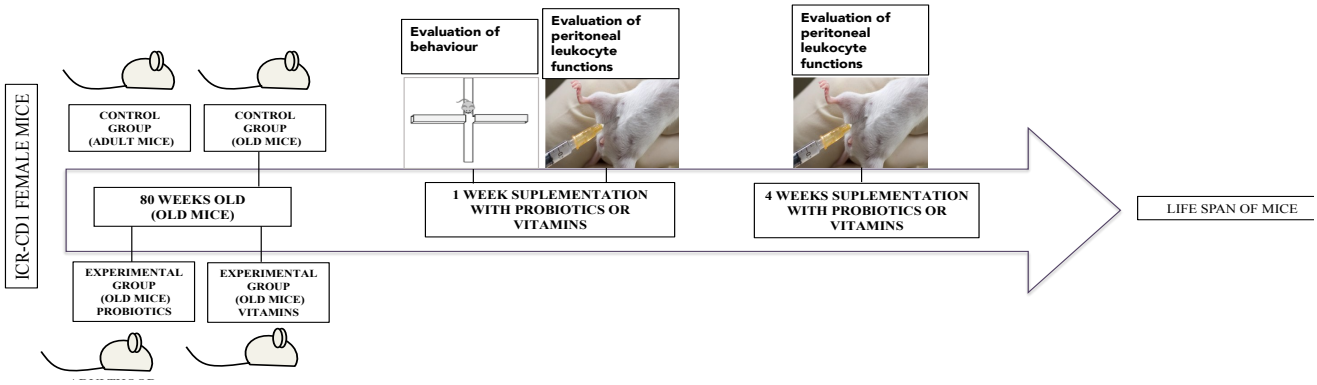
3.4. EFFECTS OF THE DIETARY SUPPLEMENTATION WITH FERMENTED MILK CONTAINING PROBIOTICS DURING DIFFERENT PERIODS OF TIME ON BEHAVIOUR, IMMUNE FUNCTION AND REDOX STATE OF OLD MICE, AS WELL AS ON THEIR LIFE SPAN.

3.4.1. Effects of the dietary supplementation with fermented milk containing probiotics for one and four weeks on behaviour and immune function of old female mice, as well as on their life span



Experimental design

Old mice (80 weeks old) were separated into three groups (n=8-10, per group). 1) Old mice without dietary supplementation. 2) Old mice supplemented with fermented milk containing probiotics (a commercial fermented milk drink for four weeks). 3) Old mice supplemented with skimmed milk containing the same amount of vitamins B6 and D as the commercially fermented milk for four weeks. An additional group of adult mice (n=8-10) was used as an age control. The commercial fermented milk drink studied contains two bacterial strains commonly used in yogurt, *Lactobacillus delbrueckii* subsp. *Bulgaricus* and *Streptococcus thermophiles*, and the probiotic *Lactobacillus casei* DN114001 (10^9 CFU/100g). The vitamins B6 and D were also present (0.21 mg/100g and 0.75µg/100g, respectively). Weight, food and supplementation intakes were measured every day. After one week of dietary supplementation with fermented milk (probiotics) or skimmed

milk (vitamins), a variety of tests and assays were performed in order to evaluate the behaviour of animals. Peritoneal leukocytes were also obtained for the analysis of several functions. Given that mice are capable of remembering recently performed behavioural tests, only the immunological functions were studied after four weeks of dietary supplementations. Furthermore, in parallel with these groups, another group of old mice (n=5) was used in order to test the *in vitro* effect of probiotic, vitamins and other components, present in the fermented milk, on several functions of peritoneal leukocytes.

Main results

Body weight, food and drink intake measurements

There were no significant differences in the body weight after four weeks of dietary supplementation. Thus, old mice supplemented with fermented milk (probiotics) exhibited an average weight of 42 ± 6 g, with skimmed milk (vitamins) of 44 ± 6 g and without supplementation of 42 ± 7 g. Also, there were no significant differences in the average daily food intake during the supplementation period among the three groups of old mice (5 ± 1 g/day/mouse, in old mice supplemented with fermented milk, 5 ± 1.4 g/day/mouse, in old mice supplemented with skimmed milk and 5 ± 1g/day/mouse, in old non-supplemented controls). However, with respect to the average daily water intake, old non-supplemented controls ingested a significantly higher amount of water (10 ± 3 ml/day/mouse) than old mice supplemented with fermented milk (7 ± 2 ml/day/mouse) and with skimmed milk (6 ± 3 ml/day/mouse). The average daily supplementation intake during four weeks was 8 ml/day/mouse, for the group of old mice supplemented with fermented milk and 9 ml/day/mouse, for old mice supplemented with skimmed milk.

Behavioural parameters

Reflexes

The hindlimb extensor reflex was significantly lower in old controls than in adult controls. Old mice supplemented with fermented milk (probiotics) or skimmed milk (vitamins B6 and D) for

one week exhibited a significantly higher performance of the hindlimb extensor reflex than old non-supplemented controls. No significant differences were found among the groups of mice studied regarding the performance of the visual placing reflex.

Motor coordination and equilibrium

In the wood rod test, old controls showed significantly impaired motor coordination and equilibrium abilities in comparison with adult controls. However, dietary supplementation for one week with fermented milk (probiotics) or with skimmed milk (vitamins) resulted in a significantly lower percentage of old animals falling off from the rod than old non-supplemented controls. In addition, the percentage of old mice supplemented with fermented milk that covered at least 1 segment and that completed the test, was significantly higher in comparison with old non-supplemented controls and old mice supplemented with skimmed milk.

Muscular vigour and traction

In the tightrope test, old controls showed significantly impaired muscular vigour and traction capacities in comparison with adult controls. However, the supplementation with fermented milk for one week was able to diminish the percentage of mice that fell off the rope compared with old non-supplemented controls and old mice supplemented with skimmed milk. With respect to the percentage of mice that covered at least 1 segment and that completed the test, old mice supplemented with fermented milk (probiotics) or with skimmed milk (vitamins) displayed significantly better results than old non-supplemented controls. In addition, both supplementations resulted in maximum traction capacity in old mice in comparison with old non-supplemented controls. However, old mice supplemented with fermented milk showed significantly better response in all parameters that evaluated muscular vigour and traction than old mice supplemented with skimmed milk.

Vertical exploratory activity

The vertical exploratory activity of mice was assessed by the frequency of rearing. The percentage of old controls that performed rearings in the corner test and in the holeboard test was

significantly lower than in adult controls. The one-week supplementation with fermented milk resulted in significantly higher percentage and number of rearings in the corner test and in the holeboard test in comparison with old non-supplemented controls. In addition, in the holeboard test, there was a higher percentage of old mice supplemented with skimmed milk that performed rearings in comparison with old non-supplemented controls. Nevertheless, old mice supplemented with fermented milk showed a higher percentage of rearings performed in the corner test and in the holeboard test than old mice supplemented with skimmed milk.

Horizontal exploratory activity

In the corner test, old mice supplemented with fermented milk for one week displayed a higher number of corners visited than old non-supplemented controls. With respect to central locomotion in the holeboard test, old mice supplemented with fermented milk exhibited a significantly higher locomotion than old non-supplemented controls and old mice supplemented with skimmed milk. There were no significant differences in the total and in the peripheral locomotion among the groups of mice studies in the holeboard test. In addition, no significant differences were observed among the groups of animals in the performance of the T-maze test.

Goal-directed exploratory behaviour

The goal-directed exploratory behaviour of mice was significantly lower in old controls with respect to adults. However, the one-week supplementation with fermented milk or with skimmed milk resulted in a higher percentage of old mice performing head-dips than old non-supplemented controls. In addition, old mice supplemented with fermented milk performed more head-dips than old mice supplemented with skimmed milk.

Behaviours related to anxiety-like behaviour

Old controls showed a higher frequency performing self-grooming in the holeboard test and in the T-maze test than adult controls. The one-week supplementation with fermented milk or with skimmed milk resulted in a lower percentage of old mice performing self-grooming in the T-maze test in comparison with old non-supplemented controls. In addition, in the holeboard test, old mice

supplemented with fermented milk showed a lower number of self-groomings with respect to old non-supplemented controls. The presence of fecal bolei in the T-maze test was significantly higher in old controls in comparison with adult controls. The supplementation with fermented milk or with skimmed milk for one week resulted in a significantly lower presence of fecal bolei than old non-supplemented controls.

Peritoneal leukocyte function parameters

The chemotaxis index of macrophages (mobility in response to a chemotactic gradient such as formylated peptide) was significantly suppressed in old mice compared with adult mice. Old mice supplemented with fermented milk (probiotics) for one and four weeks displayed significantly higher values of this chemotaxis index in comparison with old non-supplemented controls and old mice supplemented with skimmed milk. No significant differences were observed between old mice supplemented with skimmed milk (one and four weeks) and old non-supplemented controls.

The phagocytic index was significantly lower in old mice with respect to adult mice. The supplementation with fermented milk (probiotics) in old mice, at different times (one and four weeks), resulted in a higher phagocytic index than in old non-supplemented controls. The supplementation with skimmed milk (vitamins) was not able to improve this function in comparison with old non-supplemented controls. However, a significantly higher phagocytic index in the group of old mice supplemented with skimmed milk (vitamins) for four weeks was observed with respect to the same group supplemented for one week. The number of macrophages with phagocytic ability (phagocytic efficiency) was significantly lower in old controls than in adult controls. Old mice supplemented with fermented milk (probiotics) for one and four weeks or with skimmed milk (vitamins) for four weeks showed significantly higher phagocytic efficiency than old non-supplemented animals. In addition, old mice supplemented with fermented milk (probiotics) for four weeks exhibited higher phagocytic efficiency in comparison with old mice supplemented with skimmed milk (vitamins). There were no statistically significant differences between old mice supplemented with skimmed milk (vitamins) for one week and old non-supplemented controls.

However, old mice supplemented with skimmed milk for four weeks displayed significantly higher values of phagocytic efficiency than the same group of old mice supplemented for one week.

The chemotaxis index of peritoneal lymphocytes induced by a chemotactic peptide showed significantly lower values in old mice than in adult mice. The one and four weeks supplementation with fermented milk (probiotics) in old mice resulted in significantly higher chemotaxis index than in non-supplemented old mice and old mice supplemented with skimmed milk (vitamins). Furthermore, old mice supplemented with skimmed milk for four weeks showed a higher chemotaxis index of lymphocytes when compared with old non-supplemented controls. No significant differences were found between old mice supplemented with skimmed milk for one week and old non-supplemented controls.

The anti-tumour NK activity was significantly suppressed in old mice in comparison with adult mice. Old mice supplemented with fermented milk (probiotics) at different times (one and four weeks) showed significantly higher NK activity than old non-supplemented controls. In addition, old mice supplemented with skimmed milk (vitamins) for four weeks resulted in higher NK activity than old non-supplemented controls. However, old mice supplemented with skimmed milk (vitamins) for one week did not show significant differences in comparison with old non-supplemented controls.

The lymphoproliferation in response to T-cell mitogen (ConA) and to B-cell mitogen (LPS) was lower in old mice than in adults. Old mice supplemented with fermented milk (probiotics) for four weeks displayed significantly higher proliferation of lymphocytes in response to ConA and LPS than old non-supplemented controls. In addition, in the group of old mice supplemented with skimmed milk for four weeks a higher proliferation of lymphocytes in response to LPS in comparison with old non-supplemented controls was observed. Nevertheless, old mice supplemented with skimmed milk for four weeks did not show significant differences in the proliferation of lymphocytes in response to ConA in comparison with old non-supplemented controls. Furthermore, old mice supplemented with fermented milk or with skimmed milk for one

week showed no significant differences in the proliferation of lymphocytes in response to ConA or LPS.

In vitro immunoassays

The chemotaxis index of lymphocytes with the presence of fermented milk supernatant, which contains probiotic bacteria, showed significantly higher values than in controls. In addition, the chemoattractant capacity of fermented milk supernatant tended to be higher than the chemoattractant capacity of the formyl peptide. With respect to antitumour NK activity, the fermented milk supernatant added to the effector cells (leukocytes) showed significantly higher values than in controls.

Life span

The differences were not statistically significant among the groups of mice. The average of life span of old mice supplemented with fermented milk containing probiotics was 108 ± 10 weeks, with skimmed milk (vitamins B6 and D) it was 100 ± 15 weeks, and in old non-supplemented controls it was 111 ± 15 weeks.

Partial conclusions

The results of this experiment indicate that a short-term (one week) supplementation with a commercial drink containing probiotics is able to improve behaviour and immune function of old mice, these reaching similar values to those found in adult controls. Furthermore, a longer-term supplementation (four weeks) maintains these improvements in the same immune function parameters.

Hunsche et al., J Prob Health 2018, 6:1
DOI: 10.4172/2329-8901.1000201

Journal of Probiotics & Health

Research Article Open Access

Dietary Supplementation with Fermented Milk Containing Probiotics Improves Behaviour and Immune Response of Aged Mice

Caroline Hunsche, Julia Cruces, Antonio Garrido, Oskarina Hernandez and Mónica De la Fuente*

Department of Genetics, Physiology and Microbiology, Complutense University of Madrid, Madrid, Spain

***Corresponding author:** Mónica De la Fuente, Department of Genetic, Physiology and Microbiology, Complutense University of Madrid, José Antonio Novais Street, 28040, Madrid, Spain, Tel: 0034 91 3944989; E-mail: mondelaf@bio.ucm.es

Received date: June 06, 2018; **Accepted date:** June 14, 2018; **Published date:** June 21, 2018

Abstract

Aging is associated with an impairment of the regulatory systems, including the nervous and immune systems as well as the communication between them. The consumption of fermented milk containing live probiotic bacteria seems to have neurological and immunological benefits. The aim of the present study was to evaluate the effects of the supplementation with a fermented milk containing yogurt cultures and the probiotic *Lactobacillus casei* DN-114001 on behaviour and immune functions of old mice as well as on their life span. Also, the *in vitro* properties of these probiotics on functions of peritoneal leukocytes were analysed. Old female ICR-CD1 mice were supplemented with fermented milk containing probiotics. After 1 and 4 weeks of supplementation, behavioural tests were performed and immunological functions in peritoneal leukocytes studied. The results showed that a short-term (one week) supplementation with fermented milk containing probiotics was able to improve behavioural aspects (such as motor coordination, equilibrium, muscular vigour, exploratory activity and anxiety-related behaviour) as well as immune functions in old mice that had been impaired by age, reaching similar levels to those found in adult controls. Furthermore, a longer-term supplementation (four weeks) was able to maintain these improvements in the immunological parameters. Probiotics seem to have a direct effect on several immune functions. In conclusion, the supplementation with fermented milk containing probiotics could be a good nutritional strategy to improve behaviour and immune system functions in old individuals and, consequently, to promote healthier aging.

Keywords: Aging; Fermented milk with probiotics; Behaviour; Immune system functions

Introduction

The impairment in aging of the immune and nervous systems has been associated with increased susceptibility to infectious diseases, cancer, and neurodegenerative diseases [1]. It is well known that the nervous and immune systems are intricately connected and share a bidirectional communication. Thus, any alteration exerted on the immune system will promote an effect on the nervous system and vice versa [1,2]. With respect to the central nervous system, there is an age-related progressive deterioration of a variety of brain and behavioural functions, including memory, cognitive, and motor activity [3,4]. Moreover, old individuals become more susceptible to suffer mental disorders, such as anxiety [5]. Also, the immune system undergoes a variety of changes, denominated immunosenescence, which include altered innate and adaptive immune functions, such as a marked decreased activity of phagocytic cells, anti-tumour natural killer (NK) cytotoxicity and proliferation of lymphocytes [6]. Increasing evidence suggests that the age-related deterioration of the nervous and immune systems are mainly based on oxidative and inflammatory stresses (an imbalance between oxidant and pro-inflammatory compounds versus antioxidant and anti-inflammatory compounds in favour of the former). Although the production of low levels of oxidants and inflammatory compounds is essential for effective immune response, an overproduction of these compounds by immune cells may lead to cellular and tissue damages [7]. In fact, an oxidative-inflammatory theory has been proposed to explain the cause of aging. Moreover, in this theory, the immune system has been described as a critical player in these stresses and consequently in the rate of aging [8].

The composition and functionality of gut microbiota are also affected by aging, the gut microbiota diversity of aged individuals being lower compared with younger people [9,10]. There is also a decrease in beneficial species, such as *Bifidobacteria* [11]. In this sense, the consumption of fermented milk containing probiotics has gained much attention from scientific and commercial communities [12]. Probiotics are live microorganisms that are claimed to confer health benefits when administered in adequate amounts [13]. Many reports have indicated that certain strains of probiotics are capable of stimulating and modulating the immune response of the host. Thus, there is an increased application of probiotics to treat immune impairments, such as allergic, inflammatory and autoimmune disorders [14-18]. Several studies also indicated anti-aging (ameliorating immunosenescence) [19] and anti-tumour proprieties of probiotics [20]. In addition, growing evidence suggest that probiotics could also affect brain and behavioural functions [21-25]. It seems that probiotics through the modulation of gut microbiota, or directly, could beneficially affect the bidirectional communication between gut and brain, from which has emerged the concept of microbiota-gut-brain axis [21].

Many studies have been conducted in order to analyse the immune modulating properties of probiotics in adults, and several on their behavioural effects. However, little research has been developed to study particularly the repercussion of probiotics on these aspects in older individuals, as well as on life spans, for which is necessary to use experimental animals with very shorter longevity than humans. Thus, the aim of the present work was to evaluate the effects of the

supplementation with a fermented milk containing yogurt cultures and the probiotic *Lactobacillus casei* DN-114001 on behaviour and immune functions of old mice as well as on their life span. The *in vitro* properties of these probiotics on some functions of peritoneal leukocytes were also analysed to detect possible direct effects.

Materials and Methods

Animals

Old (80 ± 4 weeks of age) and adult (32 ± 4 weeks of age) female ICR/CD1 mice (*Mus musculus*) (Janvier SAS, Chassal, Francia) were used. The animals were housed in polyurethane cages (5 animals per cage) and maintained under standard laboratory conditions (12:12 h reversed light/dark cycle; lights on at 8:00 pm, relative humidity of 50-60%, temperature of 22 ± 2°C and adequate ventilation). All mice were fed a standard maintenance diet (Teklad Global 14% Protein Rodent Maintenance Diet, Harlan Interfauna Iberica, Barcelona, Spain) and water at libitum. The experiments were conducted in accordance with the guidelines and protocols of the Royal Decree 53/2013 regarding the care and use of laboratory animals for experimental procedures, and were approved by the Committee for Animal Experimentation of the Complutense University of Madrid.

Experimental groups

Old mice were separated into three groups (n=8-10, per group), namely (1) old mice which were not dietary supplemented; (2) old mice supplemented with fermented milk containing probiotics (a commercial fermented milk drink during four weeks); (3) old mice supplemented with skimmed milk containing the same amount of vitamins B6 and D as the commercially fermented milk for four weeks. An additional group of adult mice (n=8-10) was used as age controls. The commercial fermented milk drink studied contains two bacterial strains commonly used in yogurt, *Lactobacillus delbrueckii subsp. Bulgaricus* and *Streptococcus thermophilus*, and the probiotic *Lactobacillus casei* DN114001 (10^9 CFU/100 g). The vitamins B6 and D were also present (0.21 mg/100 g and 0.75 μg/100 g, respectively). Weight, food and supplementation intakes were measured every day. After one week of dietary supplementation with ferment milk (probiotics) or skimmed milk (vitamins), a variety of tests and assays were performed in order to evaluate the behaviour of animals and peritoneal leukocytes were obtained for the analysis of several functions. Given that mice are capable of remembering recently performed behavioural tests, only the immunological functions were studied after four weeks of dietary supplementations. Furthermore, in parallel with these groups, another group of old mice (n=5) was used in order to test the *in vitro* effect of probiotic, vitamins and other components, present in the fermented milk, on several functions of peritoneal leukocytes.

Fermented milk supernatant for the *in vitro* immunoassays

A sample of the fermented milk drink was centrifuged at 3600 g for 10 minutes at 4°C and two successive centrifugations under the same conditions were performed from the resulting supernatant. The supernatant, composed of microbial probiotic strains (*Lactobacillus delbrueckii subsp. Bulgaricus*, *Streptococcus thermophilus*, *Lactobacillus casei* DN114001), vitamins and other components of the fermented milk drink, was used to evaluate the chemotaxis capacity of lymphocytes and the natural killer cytotoxicity, as well as assessing its chemoattractant activity.

Behavioural tests

Behavioural tests were carried out between 8 am and 10 am during two consecutive days, followed a procedure previously described [2]. On the first day, a battery of tests (reflexes, T-maze, wood rod, corner and tightrope tests) was performed. On the second day, mice were subjected to the holeboard test. The experimental apparatus was cleaned before the performance of each mouse in order to avoid olfactory trails.

Reflexes: Visual and hindlimb extensor reflex: When the mouse was suspended by the tail and lowered toward a solid black surface, the visual placing reflex was evaluated by observing the complete extension of the forelimbs, and the hindlimb reflex was measured by the complete extension of these limbs. The mean response was rated in three trials.

Motor coordination and equilibrium abilities: In order to assess motor coordination and equilibrium, animals were placed in the centre of a wood elevated rod (rod dimensions: 22 cm height, 80 cm length, 2.9 cm width, divided in segments of 10 cm) for one trial of 60 s. Motor coordination and equilibrium were measured by the percentage of mice falling off the rod. In addition, the percentages of mice that cover at least 1 segment and that complete the test were also considered as motor coordination and equilibrium abilities.

Muscular vigour and traction: As an index of muscular vigour, mice were hanged by its forelimbs in the middle of an elevated horizontal tightrope (40 cm height, 60 cm length and divided into 6 segments of 10 cm) [2]. This method is used to evaluate the muscular vigour and traction in one training trial of 5 seconds and a test trial of 60 seconds. Muscular vigour was assessed as the percentage of mice falling off the rope and as the percentages of mice that cover at least 1 segment and that complete the test. Traction was evaluated by analysing the different parts of the body that mice used to remain hanged (forelimbs, hindlimbs and tail) and, subsequently, the percentages of mice displaying the maximum traction capacity (using forelimbs, hindlimbs and tail).

Non-goal-directed: Behaviour vertical exploratory activity: The vertical exploratory activity (studied as the performance of "rearing", that is, when the mouse stands up on his hindlimbs so that his body becomes more perpendicular to the ground) was evaluated in the corner test and in the holeboard test. The corner test consists in placing the mouse in a new cage (22.0 × 22.0 × 14.5 cm) with bedding during 30 s. In contrast, the holeboard test consists in placing the animal in a box made of wood (60 × 60 × 45 cm) with matte-painted metallic walls, divided into 36 squares (10 × 10 cm), bearing four equally spaced holes (3.8 cm of diameter). All but 20 peripheral squares were considered central. The test was performed during 5 minutes [2].

Horizontal exploratory activity: Horizontal exploratory activity was assessed as the number of corners of the cage visited (in the corner test) and as the peripheral locomotion (number of line crossings in the peripheral area, by the walls) and central locomotion (number of line crossings in the central area) in the holeboard test. In addition, horizontal exploratory behaviour was also recorded using the T-maze test, which is a wooden apparatus with three enclosed arms (short arm: 27 × 10 cm; long arm: 64 × 10 cm; walls: 19 cm high) [2]. The mouse was placed inside the "short" arm of the maze with its head facing the wall. The time elapsed (in seconds) until the animal crosses with both

hindlimbs the intersection of the T-maze and the time (in seconds) to explore the entire maze (exploration of all three maze arms) were assessed.

Goal-directed exploratory behaviour: The goal-directed exploratory behaviour of mice was assessed in the holeboard test. An object formed by a white plastic was placed into each of the four holes of the holeboard to attract mice attention. The frequency (percentage and number) of mice that perform head-dips (when the animal places its head into the hole) was considered as a goal-directed exploratory behaviour.

Behaviours related to anxiety-like behaviour: Behaviour related to anxiety-like behaviour was measured in holeboard and T-maze tests. The total number and percentage of mice that perform self-grooming (when mouse cleans, licks or scratches its body) in both tests and the presence of defecation in the T-maze test were recorded. This last parameter was also considered as an index of anxiety-like behaviour.

Collection of peritoneal leukocytes

The peritoneal suspensions were obtained between 8 am and 10 am to minimize circadian variations in the immune system, by a procedure previously described, without animal sacrifice [26,27], which allowed monitoring the life span of the mice. Briefly, 3 ml of Hank´s solution, adjusted to pH 7.4, were injected into the peritoneum, the abdomen was massaged and the peritoneal exudate cells were collected allowing the recovery of 90-95% of the injected volume. The peritoneal leukocytes, consisting of lymphocytes and macrophages, were counted in Neubauer chambers (Blau Brand, Germany). The suspensions were adjusted to a final concentration of 5×10^5 macrophages or lymphocytes/ml in Hank´s solution or 10^6 leukocytes/ml in Hank's solution or complete medium (RPMI 1640 enriched with L-glutamine (PAA, Pasching, Austria) and supplemented with 10% heat-inactivated (56°C, 30 min) fetal calf serum (PAA) and gentamicin (100 mg/ml, PAA) with or without phenol red, depending on the type of assay used. Macrophages and lymphocytes were identified by their morphology. The cellular viability was measured using the trypan-blue (Sigma-Aldrich, Madrid, Spain) exclusion test and in all cases was higher than 98%.

The immune functions parameters studied were performed using unfractionated peritoneal leukocytes, which better preserved the physiological environment surrounding the immune cells *in vivo* [28].

Chemotaxis assay: Chemotaxis of peritoneal leukocytes was evaluated according to a slight modification of Boyden's method [26], consisting basically of the use of chambers with two compartments separated by a filter with a pore diameter of 3 µm (Millipore, Bedford, MA, USA). Aliquots of 300 µl of the peritoneal suspensions, with macrophages or lymphocytes adjusted to 5×10^5 cells/ml in Hank's solution, were deposited in the upper compartment, and aliquots of 400 µl of the chemoattractant, formyl-Met-Leu-Phe (10^{-8} M) (Sigma-Aldrich), were put into the lower compartment. The chambers were incubated for 3 h and then the filters were fixed and stained. The chemotaxis index (CI) was determined by counting, in an optical microscope (100X), the total number of macrophages and lymphocytes on one third of the lower face of the filters.

Phagocytosis assay: Phagocytosis assay of inert particles (latex beads) was carried out following a method previously described [26]. Aliquots of 200 µl of the peritoneal suspensions adjusted to 5×10^5 macrophages/ml in Hank's medium were incubated in migration inhibitory factor (MIF) plates (Kartell, Noviglio, Italy) for 30 min. The adhered monolayer was washed with pre-warmed PBS (phosphate buffer saline), and then 200 µl of Hank's medium and 20 µl of latex bead suspension (1.09 µm, diluted to 1% PBS) (Sigma-Aldrich) were added. After 30 min of incubation, the plates were washed, fixed and stained. The number of particles ingested by 100 macrophages was counted using an optical microscope (100X) and expressed as phagocytic index (PI). The percentage of macrophages, which phagocyted at least one latex bead, was also determined and expressed as phagocytic efficiency (PE).

Natural killer assay: An enzymatic colorimetric assay was carried out to measure the cytolysis of tumour cells (Cytotox 96 TM Promega, Boehringer Ingelheim, Germany) based on the determination of lactate dehydrogenase enzyme (LDH), as previously described [26]. Aliquots of 100 µl of peritoneal leukocytes, used as effector cells, were seeded in 96-well U-bottom culture plates (Numc, Roskilde, Denmark) adjusted to 10^6 leukocytes per ml in RPMI 1640 medium without phenol red. Murine lymphoma YAC-1 cells, used as target cells, were added adjusted to 10^5 cells per ml. Thus, the effector/target ratio was 10:1. The plates were centrifuged at 250 g for 4 min to facilitate cell contacts. After 4 hours of incubation, lactate dehydrogenase enzymatic activity was measured in 50 µl/well of the supernatants by addition of the enzyme substrate and absorbance recording spectrophotometrically at 490 nm. Three kinds of control measurements were performed: a target spontaneous release, a target maximum release, and an effector spontaneous release. The results were expressed as percentage of lysis of target cells. To determine this percentage the following equation was used: %lysis=((E-ES-TS)/M-ES-TS)) × 100, where E is the mean of absorbance in the presence of effector cells; ES, the mean of absorbance of effector cells incubated alone; TS, the mean of absorbance in target cells incubated with medium alone; and M is the mean of maximum absorbance after incubating target cells with lysis solution.

Lymphoproliferation assay: Following the method previously described [27], aliquots (200 µl) of peritoneal leukocytes (10^6 lymphocytes/ml complete medium) were seeded in 96 well flat-bottomed microtitre plates (Numc, Roskilde, Denmark) and 20 µl of concanavaline A (ConA 1 µg/ml; Sigma-Aldrich), 20 µl of lipopolysaccharide (LPS, *E. coli*, 055:B5 1 µg/ml; Sigma-Aldrich) or 20 µl of complete medium (spontaneous proliferation) were added per well. After 48 h of incubation at 37°C in an atmosphere of 5% CO_2, 0.5 µCi ^{3}H-thymidine (Du Pont, Boston, MA, USA) were added to each well. The cells were harvested in a semiautomatic microharvester 24 h later. Thymidine uptake was measured using a beta counter (LKB, Uppsala, Sweden). The results were expressed as ^{3}H-thymidine uptake (cpm).

In vitro immunoassays for testing microbial probiotic strains and other components of fermented milk

Chemotaxis assay: The chemotaxis index of lymphocytes was assessed as previously described above. However, aliquots of 300 µl of the peritoneal suspensions adjusted to 5×10^5 lymphocytes/ml in Hank's solution were added to 30 µl of Hank's solution (control) or to 30 µl of fermented milk supernatant and then deposited in the upper compartment of the chamber. Also, for evaluation of the chemoattractant capacity of lymphocytes, 400 µl of the fermented milk supernatant or aliquots of the chemoattractant, formyl-Met-Leu-Phe (10^{-8} M) (Sigma-Aldrich) were put into the lower compartment of the chamber. The chemotaxis index (CI) was determined by counting, in

an optical microscope (100X), the total number of lymphocytes on one third of the lower face of the filters.

Natural killer assay: An enzymatic colorimetric assay was carried out to measure the cytolysis of tumour cells (Cytotox 96 TM Promega, Boehringer Ingelheim, Germany) as previously described above. Nevertheless, it was used as effector cells aliquots of 100 µl of peritoneal leukocytes (adjusted to 10^6 leukocytes/ml) with 10 µl of fermented milk supernatant. Murine lymphoma YAC-1 cells (target cells) were added adjusted to 10^5 cells/ml. The results were expressed as percentage of lysis of target cells.

Statistical analysis: SPSS 21.0 (SPSS, Inc., Chicago, USA) was used for the statistical analysis of the results. The data were expressed as mean ± standard deviation (SD). Each value is the mean of the data from an assay performed in duplicate or triplicate. Normality of the samples was checked by the Kolmogorov-Smirnov test and homogeneity of variances with the Levene test. The data were statistically evaluated by the Student's t-test for independent and paired samples. $P<0.05$ was considered statistically significant and $0.05<P<0.1$ was considered as a trend.

Results

Body weight, food and drink intakes

There were no significant differences in the body weight after four weeks of dietary supplementation. Thus, old mice supplemented with fermented milk (probiotics) exhibited an average weight of 42 ± 6 g, with skimmed milk (vitamins) of 44 ± 6 g and without supplementation of 42 ± 7 g. Also, there were no significant differences in the average daily food intake during supplementation period among the three groups of old mice (5 ± 1 g/day/mouse, in old mice supplemented with fermented milk, 5 ± 1.4 g/day/mouse, in old mice supplemented with skimmed milk and 5 ± 1 g/day/mouse, in old non-supplemented controls). However, with respect to the average daily water intake, old non-supplemented controls ingested a significant higher amount of water (10 ± 3 ml/day/mouse) than old mice supplemented with fermented milk (7 ± 2 ml/day/mouse) and with skimmed milk (6 ± 3 ml/day/mouse) ($P<0.001$). The average daily supplementation intake during four weeks was 8 ml/day/mouse, for the group of old mice supplemented with fermented milk and 9 ml/day/mouse, for old mice supplemented with skimmed milk.

Behavioural tests

Table 1 shows the results obtained in the behavioural assessments of old non-supplemented mice and old mice supplemented with fermented milk (probiotics) or skimmed milk (vitamins).

Behaviour	Old mice			
	1 week supplementation			
	Adult crtl	Old crtl	Fermented milk with probiotics	Skimmed milk with vitamins
Reflexes				
% of mice that show visual placing reflex	100	100	100	100
% of mice that show hindlimb extensor reflex	100***	86	100***	100***
Motor coordination and equilibrium (Wood rod test)				
% of mice falling off	0***	29	14***	12***
% of mice that cover at least 1 segment	100***	71	100***###	75
% of mice that complete the test	100***	57	86***###	62
Muscular vigour (Tightrope test)				
% of mice falling off	14***	87	71**##	87
% of mice that cover at least 1 segment	100***	0	57***##	37***
% of mice that complete test	87***	0	29***##	12**
Traction (Tightrope test)				
% of mice that show maximum traction capacity	87***	14	57***##	37***
Non-goal-directed exploratory behaviour				
Vertical exploratory activity				
% of mice that perform rearing (Corner test)	100***	86	100***###	87

Total number of rearings (Corner test)	5 ± 1.5	3.5 ± 2.5	6 ± 1.5*	4.5 ± 2.5
% of mice that perform rearing (Holeboard test)	86*	71	100***##	88**
Horizontal exploratory activity				
Number of corners visited (Corner test)	7 ± 1.5	8.5 ± 2	11 ± 1.5*	9.5 ± 4
Total number of line crossings (Holeboard test)	217 ± 30	222 ± 50	267 ± 74	229 ± 96
Number of line crossings in the peripheral area (Holeboard test)	118 ± 26	137 ± 40	149 ± 54	148 ± 74
Number of line crossings in the central area (Holeboard test)	99 ± 13	85 ± 31	118 ± 33*#	81 ± 28
Time for crossing the intersection of the maze (s) (T-maze test)	10 ± 2	10 ± 6	7 ± 4	14 ± 8
Time to complete the test (s) (T-maze test)	36 ± 9	27 ± 11	25 ± 13	48 ± 30
Goal-directed exploratory behaviour				
% of mice that perform head-dips (Holeboard test)	100*	93	100*	100*
Number of head-dips (Holeboard test)	10 ± 3	7 ± 4	10 ± 3##	5 ± 2
Behaviours related to anxiety-like behaviour				
Total number of self-grooming (Holeboard test)	1 ± 1*	4 ± 3	2 ± 0.5*	2 ± 1
% of mice that perform self-grooming (T-maze test)	0***	14	0***	0***
% fecal bolei presence (T-maze test)	0***	29	14*	12**

Table 1: Behavioural tests in old mice supplemented with fermented milk (probiotics) and with skimmed milk (vitamins) during one week. Results are expressed as percentage (%) or mean ± SD in adult control (n=8-10), old control (n=8-10), old mice supplemented with fermented milk (n=8-10) and old mice supplemented with skimmed milk (n=8-10). *P<0.05; **P<0.01; ***P<0.001 with respect to the values of old control mice. #P<0.05; ##P<0.01; ###P<0.001 with respect to the values of old mice supplemented with skimmed milk.

Reflexes: The hindlimb extensor reflex, which is shown in Table 1, was significantly lower in old controls than in adult controls (P<0.001). Old mice supplemented with fermented milk (probiotics) or skimmed milk (vitamins B6 and D) during one week exhibited a significantly higher performance of the hindlimb extensor reflex than old non-supplemented controls (P<0.001). No significant differences were found among the studied groups of mice regarding the performance of the visual placing reflex (Table 1).

Motor coordination and equilibrium: In the wood rod test, old controls showed significantly impaired motor coordination and equilibrium abilities in comparison with adult controls (P<0.001, Table 1). However, dietary supplementation during one week with fermented milk (probiotics) or with skimmed milk (vitamins) resulted in significantly lower percentage of old animals falling off from the rod than old non-supplemented controls (P<0.001, Table 1). In addition, the percentage of old mice supplemented with fermented milk that covered at least 1 segment and that completed the test was significantly higher in comparison with old non-supplemented controls and old mice supplemented with skimmed milk (P<0.001, Table 1).

Muscular vigour and traction: In the tightrope test, old controls showed significantly impaired muscular vigour and traction capacities in comparison with adult controls (P<0.001, Table 1). However, the supplementation with fermented milk during one week was able to diminish the percentage of mice that fell off from the rope compared with old non-supplemented controls and old mice supplemented with skimmed milk (P<0.01, Table 1). With respect to the percentage of mice that covered at least 1 segment and that completed the test (Table 1), old mice supplemented with fermented milk (probiotics) (P<0.001) or with skimmed milk (vitamins) (P<0.001 and P<0.01, respectively) displayed significantly better results than old non-supplemented controls. In addition, both supplementations resulted in maximum traction capacity in old mice in comparison with old non-supplemented controls (P<0.001, Table 1). However, old mice supplemented with fermented milk showed significantly better response in all parameters that evaluated muscular vigour and traction than old mice supplemented with skimmed milk (P<0.01, Table 1).

Non-goal-directed behaviour: Vertical exploratory activity: The vertical exploratory activity of mice, which was assessed by the frequency of rearing, is shown in Table 1. The percentage of old controls that performed rearings in the corner test and in the holeboard test was significantly lower than in adult controls (P<0.001 and P<0.05, respectively, Table 1). The one-week supplementation with fermented milk resulted in significantly higher frequency (percentage and number) of rearings in the corner test (P<0.001 and P<0.05, respectively) and in the holeboard test (P<0.001) in comparison with old non-supplemented controls (Table 1). In addition, in the holeboard test, there was a higher percentage of old mice supplemented with skimmed milk that performed rearings in comparison with old non-supplemented controls (P<0.01, Table 1). Nevertheless, old mice supplemented with fermented milk showed a higher percentage of rearing performance in the corner test and in the holeboard test than old mice supplemented with skimmed milk (P<0.001 and P<0.01, respectively, Table 1).

Horizontal exploratory activity: The results of horizontal exploratory activity are shown in Table 1. In the corner test, old mice supplemented with fermented milk during one week displayed a higher number of corners visited than old non-supplemented controls (P<0.05, Table 1). With respect to the central locomotion in the holeboard test, old mice supplemented with fermented milk exhibited a significantly higher locomotion than old non-supplemented controls and old mice supplemented with skimmed milk (P<0.05, Table 1). There were no significant differences in the total and in the peripheral locomotion among the studied groups of mice in the holeboard test (Table 1). In addition, no significant differences were observed among the groups of animals in the performance of the T-maze test (Table 1).

Goal-directed exploratory behaviour: The goal-directed exploratory behaviour of mice, which is shown in Table 1, was significantly lower in old controls with respect to adult controls (P<0.05). However, the one-week supplementation with fermented milk or with skimmed milk resulted in a higher percentage of old mice performing head-dips than old non-supplemented controls (P<0.05, Table 1). In addition, old mice supplemented with fermented milk performed more head-dips than old mice supplemented with skimmed milk (P<0.01, Table 1).

Behaviours related to anxiety-like behaviour: The results of the different tests that assessed behaviours related to anxiety-like behaviour of old mice are shown in the Table 1. Old controls showed a higher frequency performing self-grooming in the holeboard test and in the T-maze test than adult controls (P<0.05 and P<0.001, respectively, Table 1). The one-week supplementation with fermented milk or with skimmed milk resulted in a lower percentage of old mice performing self-grooming in the T-maze test in comparison with old non-supplemented controls (P<0.001, Table 1). In addition, in the holeboard test, old mice supplemented with fermented milk showed a lower number of self-grooming with respect to old non-supplemented controls (P<0.05, Table 1). The presence of fecal bolei in the T-maze test was significantly higher in old controls in comparison with adult controls (P<0.001, Table 1). The supplementation with fermented milk or with skimmed milk during one week resulted in significantly lower presence of fecal bolei than old non-supplemented controls (P<0.05 and P<0.01, respectively, Table 1).

Immune function parameters

Macrophage peritoneal functions: The peritoneal macrophage functions are shown in Figure 1. The chemotaxis index of macrophages in response to a chemotactic gradient (formylated peptide), which mimics the migration of immune cells towards the infection site, was significantly suppressed in old mice compared with adult mice (P<0.01, Figure 1A). Old mice supplemented with fermented milk during one and four weeks displayed significantly higher values of the chemotaxis index of peritoneal macrophages (Figure 1A) in comparison with old non-supplemented controls (P<0.05 and P<0.001, respectively) and old mice supplemented with skimmed milk (P<0.05). No significant differences were observed between old mice supplemented with skimmed milk (one and four weeks) and old non-supplemented controls (Figure 1A).

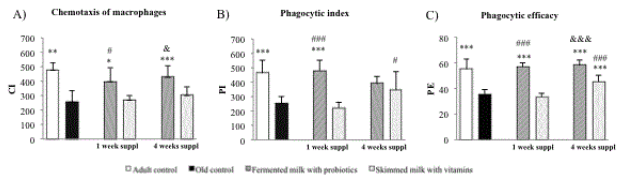


Figure 1: Macrophage functions. (A) Macrophage chemotaxis index (CI, number of macrophages). (B) Macrophage phagocytic index (PI, number latex beads/100 macrophages). (C) Macrophage phagocytic efficacy (PE, number of phagocyting macrophages/100 macrophages). Each column represents the mean ± SD of 8-10 values corresponding to that number of animals and each value being the mean of duplicate or triplicate assays. *** P<0.001; ** P<0.01 with respect to the values of old control mice. ### P<0.001; # P<0.05 with respect to the values of old mice supplemented with skimmed milk (vitamins) during one week. &&& P<0.001 with respect to the values of old mice supplemented with skimmed milk (vitamins) during four weeks.

The number of latex beads ingested by macrophages, which is measured by the phagocytic index (Figure 1B), was significantly lower in old mice with respect to adult mice (P<0.001). The supplementation with fermented milk (probiotics) in old mice, at different times (one and four weeks), resulted in a higher phagocytic index than old non-supplemented controls (P<0.001, Figure 1B). The supplementation with skimmed milk (vitamins) was not able to improve this function in comparison with old non-supplemented controls (Figure 1B). However, it was observed a significantly higher phagocytic index in the group of old mice supplemented with skimmed milk (vitamins) during four weeks with respect to the same group supplemented during one week (P<0.05, Figure 1B). The number of macrophages with phagocytic ability (Figure 1C) was significantly lower in old controls than in adult controls (P<0.001, Figure 1C). Old mice supplemented with fermented milk (probiotics) during one and four weeks or with skimmed milk (vitamins) during four weeks showed significantly higher phagocytic efficiency than old non-supplemented controls (P<0.001, Figure 1C). In addition, old mice supplemented with fermented milk (probiotics) during four weeks exhibited higher phagocytic efficiency in comparison with old mice supplemented with skimmed milk (vitamins) (P<0.001, Figure 1C). There were no statistically significant differences between old mice supplemented with skimmed milk (vitamins) during one week and old non-supplemented controls (Figure 1C). However, old mice supplemented with skimmed milk during four weeks displayed significantly higher values of phagocytic efficiency than the same group of old mice supplemented during one week (P<0.001, Figure 1C).

Lymphocyte peritoneal functions: The lymphocyte functions are displayed in Figure 2. The chemotaxis index of peritoneal lymphocytes induced by a chemotactic peptide (Figure 2A) showed significantly lower values in old mice than in adult mice (P<0.05). The one and four weeks supplementation with fermented milk (probiotics) in old mice resulted in significantly higher chemotaxis index (Figure 2A) than in old mice non-supplemented (P<0.05 and P<0.001, respectively) and old mice supplemented with skimmed milk (vitamins) (P<0.05). Furthermore, old mice supplemented with skimmed milk during four weeks showed higher chemotaxis index of lymphocytes when

compared with old non-supplemented controls (P<0.01, Figure 2A). No significant differences were found between old mice supplemented with skimmed milk during one week and old non-supplemented controls (Figure 2A).

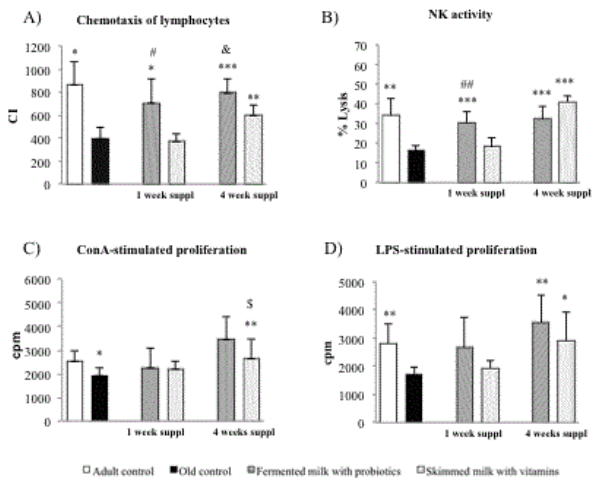


Figure 2: Lymphocyte functions. (A) Lymphocyte CI (number of lymphocytes). (B) Natural killer cell activity (% lysis). (C) Lymphoproliferative response to concanalvaline A (ConA) (cpm). (D) Lymphoproliferative response to lipopolysaccharide (LPS) (cpm). Each column represents the mean ± SD of 8-10 values corresponding to that number of animals and each value being the mean of duplicate or triplicate assays. *** P<0.001; ** P<0.01; *P<0.05 with respect to the values of old control mice. ## P<0.01; # P<0.05 with respect to the values of old mice supplemented with skimmed milk (vitamins) during one week. & P<0.05 with respect to the values of old mice supplemented with skimmed milk (vitamins) during four weeks. $ P<0.05 with respect to the values of old mice supplemented with fermented milk (vitamins) during one week.

The antitumour NK activity (Figure 2B) was significantly suppressed in old mice in comparison with adult mice (P<0.01). Old mice supplemented with fermented milk (probiotics) at different times (one and four weeks) showed significantly higher NK activity than old non-supplemented controls (P<0.001, Figure 2B). In addition, old mice supplemented with skimmed milk (vitamins) during four weeks resulted in higher NK activity than old non-supplemented controls (P<0.001, Figure 2B). However, old mice supplemented with skimmed milk (vitamins) during one week did not show significant differences in comparison with old non-supplemented controls (Figure 2B).

The lymphoproliferation in response to T-cell mitogen (ConA) and to B-cell mitogen (LPS) (Figure 2C and 2D) was suppressed in old mice with respect to adult mice (P<0.05 and P<0.01, respectively). Old mice supplemented with fermented milk (probiotics) during four weeks displayed significantly higher proliferation of lymphocytes in response to ConA (P<0.01, Figure 2C) and LPS (P<0.01, Figure 2D) than old non-supplemented controls. In addition, it was observed in the group of old mice supplemented with skimmed milk during four weeks higher proliferation of lymphocytes in response to LPS in comparison with old non-supplemented controls (P<0.05, Figure 2D). Nevertheless, old mice supplemented with skimmed milk during four weeks did not show significant differences in the proliferation of lymphocytes in response to ConA in comparison with old non-supplemented controls (Figure 2C). Furthermore, old mice supplemented with fermented milk or with skimmed milk during one week showed no significant differences in the proliferation of lymphocytes in response to ConA or LPS (Figures 2C and 2D).

In vitro immunoassays: Figure 3 shows the results from the *in vitro* immunoassays for testing microbial probiotic strains and other components of fermented milk drink. The chemotaxis index of lymphocytes with the presence of fermented milk supernatant, which contains probiotic bacteria, showed significantly higher values than control (P<0.05, Figure 3A). In addition, the chemoattractant capacity of fermented milk supernatant tended to be higher than the chemoattractant capacity of the formyl peptide (P=0.07, Figure 3B). With respect to antitumour NK activity, the fermented milk supernatant added to the effector cells (lymphocytes) showed significantly higher values than control (P<0.05, Figure 3C).

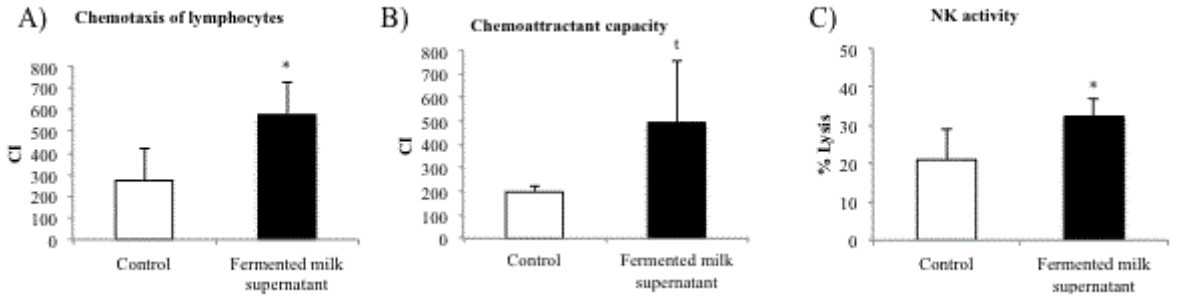


Figure 3: *In vitro* immunoassays for testing fermented milk supernatant. (A) Lymphocyte chemotaxis index (CI, number of lymphocytes). (B) Chemoattractant capacity (CI, number of lymphocytes). (C) Natural killer cell activity (% lysis). Each column represents the mean ± SD of 5 values corresponding to that number of animals and each value being the mean of duplicate or triplicate assays. *P<0.05; t 0.05<P<0.1 with respect to the values of controls.

Life span

The differences were not statistically significant among the groups of mice. The average of life span of old mice supplemented with fermented milk containing probiotics was 108 ± 10 weeks old, with skimmed milk (vitamins B6 and D) was 100 ± 15 weeks and old non-supplemented controls was 111 ± 15 weeks old.

Discussion

The consumption of fermented milk containing yogurt cultures plus *Lactobacillus casei* DN-114001 has demonstrated several immunological and gastrointestinal benefits in the elderly population. Thus, there is evidence that this dietary supplementation is capable of reducing the duration of infections [29,30] and preventing antibiotic-associated diarrhoea in old individuals [31]. However, to our knowledge, nothing has been studied so far to show whether this specific fermented milk drink could improve behavioural and immune cell function parameters, which are impaired in older individuals. Therefore, we investigated the effects of the consumption of fermented milk with probiotics on the behaviour and peritoneal leukocyte functions of old mice, as well as their life span. The immune cell function parameters studied here have been used as predictors of health status and consequently of life span. Moreover, the age-related alterations in these parameters are similar to those observed in mice (peritoneal leukocytes) and in humans (blood leukocytes) [6].

The present study found that a short-term (one week) supplementation with fermented milk containing probiotics was able to improve behaviour parameters and immune cell functions of old mice, these reaching similar values to those found in adult controls. Furthermore, a longer-term supplementation (four weeks) was able to maintain these improvements in the same immunological parameters. Nevertheless, the consumption of fermented milk with probiotics did not produce significant differences in the life span of mice. In this sense, it is possible that this consumption at old age is not sufficient to extend the life span, although the benefits found on behaviour and immune response could indicate that this supplementation leads to healthier aging. Similar results were obtained with other lifestyle strategies such as environmental enrichment, which when started in old and adult age improved these parameters of health, but was only able to increase longevity in the latter [32].

The values of the parameters measured in the battery of behavioural tests, which evaluated the abilities of motor coordination, equilibrium, muscular vigour, exploratory activity and anxiety-like behaviour, were significantly impaired in old mice in comparison to adults. However, the one-week supplementation of fermented milk with probiotics was able to improve the performance of these behavioural parameters with respect to old non-supplemented controls. Thus, supplemented animals displayed improvements in motor coordination and equilibrium (assessed by the wood rod test) and in muscular vigour (assessed by the tightrope test). There was also significantly higher exploratory activity, given that old mice supplemented with fermented milk displayed higher percentages of rearings and total number of head-dips in the holeboard test. Moreover, they showed higher locomotion in the central area of the holeboard, which indicates higher exploratory activity and lower anxiety-like behaviour. Previous reports showed that mice tend to stay in the peripheral area (close to the walls) and avoid the central area of the holeboard, which is considered more threatening by these animals [33,34]. In addition, the self-grooming behaviour and the presence of defecation, which is usually considered an index of anxiety in mice [35,36], were significantly lower in old mice supplemented with fermented milk. Currently, there is little investigation about the effect of probiotics on the behaviour of old individuals, given that most of the studies were performed using adults. In these studies, an association was found between the supplementation of probiotics and brain function and behaviour. Thus, *Lactobacilli* (latic acid bacteria) treatment for two months affected motor behaviour and decreased astrocyte reactivity of growing rats [22]. Also, the probiotic ingestion of *Lactobacillus* and *Bifidobacterium* strains has been associated with lower anxiety in adult mice [23,24]. There is also some evidence in humans that probiotics could affect mood and cognition [25]. The mechanisms by which probiotics affect brain function and behaviour remain unclear, but some evidence suggests their association with microbial composition changes, immune activation and production of neurometabolites [21].

The fermented milk with probiotic supplementation used in the present study, was also able to improve macrophage functions of old mice, which suffer an age-related impairment [6], indicating immune-

enhancing proprieties of these probiotics. Thus, old mice with the present supplementation displayed enhanced directed migration (chemotaxis) and phagocytic capacity of peritoneal macrophages in comparison to non-supplemented old mice. In agreement, another study demonstrated that the supplementation with fermented milk containing the same probiotics, i.e. yogurt cultures and the probiotic bacteria *L. casei* DN-114001, for 14 days resulted in increased phagocytic activity in peritoneal macrophages of young mice [14]. In addition, a human-based study showed that the supplementation with the probiotic *Bifidobacterium lactis* Bi-07 was capable of enhancing phagocytosis of monocytes and granulocytes in healthy elderly individuals [15].

The NK cells, known by their ability to recognize and kill tumour cells, showed a lower activity in old animals [6]. This immune function in old mice improved after supplementation in our study. Previous research on old individuals has also shown the positive effect on NK activity of several probiotics. Thus, the ingestion of a fermented drink containing *Lactobacillus casei* Shirota for four weeks improved the NK cytotoxicity of healthy aged people [16]. Moreover, another report indicated anti-tumour effects of fermented milk with probiotic consumption [20]. This anti-tumour activity of probiotics seems to be the result of a better immune response in the host (especially due to the improvement of NK cell activity). In addition, the consumption of probiotics appears to favor the decrease of the formation of carcinogenic and mutagenic compounds [20].

The lymphocyte functions, such as migration and proliferation in response to the T cell-specific mitogen (ConA) or the B cell-specific mitogen (LPS), showed lower values in old mice than in adult mice, in agreement with previous results [6]. However, supplementation (one and four weeks) of fermented milk with probiotics led to improvements in the migration of lymphocytes. With respect to proliferation of lymphocytes in response to mitogens ConA and LPS, only a long-term supplementation (4 weeks) with probiotics increased this function in old mice. The effects of probiotics on lymphocyte functions have been more studied using adult subjects. Thus, for example, they increased the response of specific antibodies against infection [17,18]. Nevertheless, there are also several articles showing the positive effects of probiotics on old experimental animals [19, 37,38].

The improvement of the immune function studied seems to be due to the direct action of probiotics since in the experiment carried out *in vitro*, the fermented milk supernatant containing probiotics enhanced chemotaxis and NK activities. It even showed a chemoattractant capacity.

Interestingly, although with fewer improvements than that with supplementation with probiotics, the control group of old mice, which were supplemented with skimmed milk enriched with vitamins B6 and D, also exhibited some benefits in behaviour and immune cell function parameters (specially after four weeks of supplementation). These results could be expected considering that these vitamins are known by their action on the nervous and immune functions [39-42].

Conclusion

In conclusion, the present study supports the effects of fermented milk containing probiotic supplementation on the function of two major regulatory systems (the nervous and the immune systems). Thus, the consumption of fermented milk with probiotics seems to be a good nutritional strategy to improve several behavioural and immune cell function parameters, which were shown to be impaired in old individuals. For this reason, this strategy could be proposed to promote healthier aging.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements

This work was supported by grants from Ministry of Science and Innovation (BFU2011-30336), the Research Group of Madrid Complutense University (910379ENEROINN), and FIS (PI15/01787) from the Institute de Salud Carlos III-Fondo Europeo de Desarrollo Regional (ISCIII-FEDER) of the European Union.

C.H. is the recipient of a PhD fellowship from CNP-q-Brazil.

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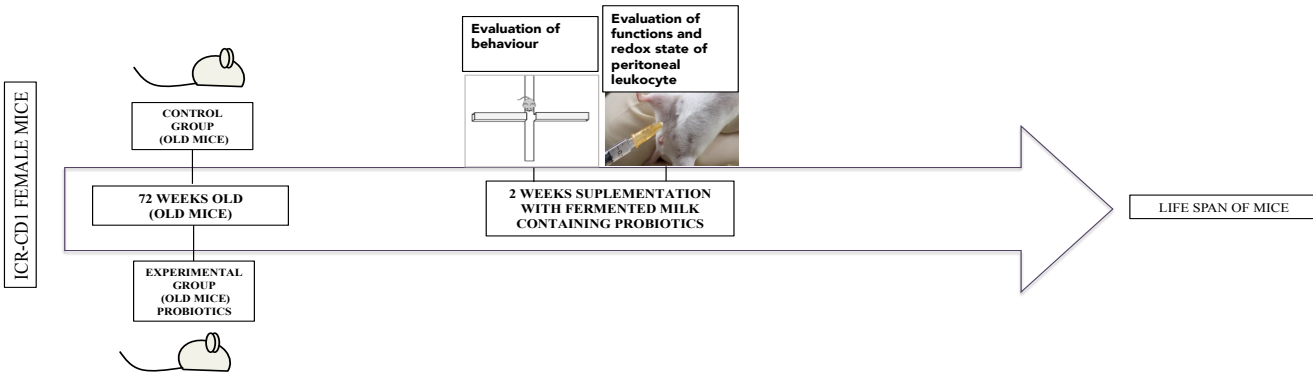
3.4.2. Effects of the dietary supplementation with fermented milk containing probiotics for two weeks on behaviour, immune function and redox state of old mice, as well as on their life span.



Experimental design

Old mice (72 weeks old) were separated according to their body weight into two groups (n=8-10, per group). 1) Old control mice without dietary supplementation. 2) Old mice supplemented with fermented milk containing probiotics for two weeks. The commercial fermented milk drink studied contains two bacterial strains commonly used in yogurt, *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophiles*, and the probiotic bacteria *Lactobacillus casei* DN114001 (10^9 CFU/100g). Weight, food and supplementation intakes were measured every day in the study. After the period of two weeks of dietary supplementation with fermented milk, a variety of tests and assays were performed on old mice in order to evaluate the behaviour of the animals, and peritoneal leukocytes were obtained for the analysis of several functions and their redox state.

Main results

Body weight, food and supplementation intake measurements

At the end of the two weeks supplementation period, old mice supplemented with fermented milk (probiotics) weighed significantly more than non-supplemented mice. However, no significant differences were observed in the average daily food intake (5 ± 0.2 g/day/mouse, in old mice supplemented with fermented milk and 5 ± 0.2 g/day/mouse, in old non-supplemented mice) and in the average daily water intake (4 ± 0.2 ml/day/mouse, in old mice supplemented with fermented milk and 5 ± 0.3 ml/day/mouse, in old non-supplemented mice). The average daily supplementation of fermented milk during the experiment period was 4 ± 1.5 ml/day/mouse.

Behavioural parameters

Motor coordination and equilibrium

In the wood rod test, no significant differences were observed in the parameters that evaluated motor coordination and equilibrium abilities between old mice supplemented with fermented milk and non-supplemented mice.

Muscular vigour and traction

In the tightrope test, old mice supplemented with fermented milk (probiotics) showed a significantly lower percentage of mice that fell off the rope than non-supplemented mice. However, the supplementation with fermented milk did not result in significant differences regarding the percentage of old mice that completed the test in comparison with old controls.

Vertical exploratory activity

The vertical exploratory activity of mice was assessed by the frequency of rearings. The percentage of old mice supplemented with fermented milk that performed rearings in the holeboard test was significantly higher than in old non-supplemented controls. In addition, in the holeboard test, the total number of rearings was significantly higher in old mice supplemented with fermented milk than in old controls.

Horizontal exploratory activity

In the corner test, no significant differences were observed in the number of corners visited between old mice supplemented with fermented milk and non-supplemented controls. The percentage of peripheral ambulation in the holeboard test was significantly lower in old mice supplemented with fermented milk in comparison with non-supplemented controls. In contrast, the percentage of central ambulation in the holeboard was significantly higher in old mice supplemented with fermented milk than in non-supplemented controls. With regards to the T-maze test, old mice supplemented with fermented milk exhibited lower times (in seconds) to explore the three arms of T-maze than old non-supplemented mice.

Goal-directed exploratory activity

No significant differences were observed in the goal-directed exploratory activity between old mice supplemented with fermented milk and old non-supplemented mice.

Anxiety-like behaviour

The supplementation with fermented milk resulted in a higher percentage of old mice performing self-grooming and in a lower percentage of old mice performing repeated digging in the corner test in comparison with non-supplemented mice. In addition, the presence of defecation in the T-maze and holeboard tests was significantly lower in old mice supplemented with fermented milk than in old non-supplemented controls.

Peritoneal leukocyte function parameters

The chemotaxis index of macrophages was significantly higher in old mice supplemented with fermented milk (probiotics) in comparison with old non-supplemented mice. In addition, the phagocytic index and the phagocytic efficiency were significantly higher in old mice supplemented with fermented milk than old non-supplemented controls.

The chemotaxis index of peritoneal lymphocytes, the NK cell activity against tumour cells and the lymphoproliferatiion in response to T-cell mitogen (ConA) and B-cell mitogen (LPS) were

significantly higher in old mice supplemented with fermented milk than in old non-supplemented animals.

Peritoneal leukocyte oxidative stress parameters

The activities of catalase (CAT) and glutathione reductase (GR) activities were significantly higher in old mice supplemented with fermented milk (probiotics) than in old non-supplemented controls. However, no significant differences were observed in the values of reduced glutathione (GSH) between old mice supplemented with fermented milk and non-supplemented controls.

Old mice supplemented with fermented milk (probiotics) exhibited lower GSSG concentrations than old non-supplemented mice. However, no significant differences were found in the GSSG/GSH ratios between old mice supplemented with fermented milk and non-supplemented animals.

Life span

The differences were not statistically significant in the life span of mice after the 2 weeks of supplementation with fermented milk. The average life span of mice supplemented with fermented milk containing probiotics was 122 ± 5 weeks, whereas in non-supplemented mice the average of life span was 118 ± 6 weeks old.

Partial conclusions

The results of this experiment indicate that the supplementation with a commercial drink containing probiotics for two weeks improves muscular vigour and exploratory activity, as well as the anxiety-like behaviour of old female mice. In addition, this supplementation results in improved function and redox state of peritoneal leukocytes in old female mice.

Current Microbiology

IMPROVEMENT OF REDOX STATE AND FUNCTIONS OF IMMUNE CELLS AS WELL AS OF BEHAVIORAL REPONSE IN OLD MICE AFTER TWO WEEKS SUPPLEMENTATION OF FERMENTED MILK WITH PROBIOTICS

--Manuscript Draft--

Manuscript Number:	CMIC-D-19-00478
Full Title:	IMPROVEMENT OF REDOX STATE AND FUNCTIONS OF IMMUNE CELLS AS WELL AS OF BEHAVIORAL REPONSE IN OLD MICE AFTER TWO WEEKS SUPPLEMENTATION OF FERMENTED MILK WITH PROBIOTICS
Article Type:	Original Paper
Funding Information:	Research Group of Madrid Complutense University (910379ENEROINN) — Mrs Caroline Hunsche Instituto de Salud Carlos III (PI15/01787) — Mrs Caroline Hunsche
Abstract:	The homeostatic systems, such as the nervous and immune systems, show deterioration in aging as consequence of the age-related oxidative-inflammatory stress establishment. The supplementation with fermented milk containing probiotic bacteria could be a good nutritional strategy to improve homeostatic system functions in aged individuals through the modulation of their redox state. The aim of the present study was to evaluate the effect of two weeks supplementation with a commercial fermented milk containing yogurt probiotics (Lactobacillus delbrueckii subsp. bulgaricus and Streptococcus thermophiles), and the probiotic Lactobacillus casei DN-114001 on behavior, redox state and immune cell functions of old mice as well as on their life span. Old female ICR-CD1 mice were supplemented with fermented milk containing these probiotics for two weeks. After this period, a variety of behavioral tests were performed and several parameters of redox state and function of peritoneal leukocytes were analyzed. The results showed that the two weeks supplementation of fermented milk containing probiotics improved behavior (such as muscular vigor, exploratory activity and anxiety-like behavior) as well as the redox state and functions of peritoneal immune cells in old mice. In conclusion, the present study shows that the supplementation with fermented milk containing probiotics for a short period of time could be a good nutritional strategy to promote healthy aging.
Corresponding Author:	Monica De la Fuente SPAIN
Corresponding Author Secondary Information:	
Corresponding Author's Institution:	
Corresponding Author's Secondary Institution:	
First Author:	Caroline Hunsche
First Author Secondary Information:	
Order of Authors:	Caroline Hunsche Julia Cruces Mónica De la Fuente
Order of Authors Secondary Information:	
Author Comments:	
Suggested Reviewers:	Teresa Requena t.requena@csic.es Similar research interest

	Natalia Ubeda nubeda@ceu.es Similar research interests
	Shima Jazayeri sh_Jazayeri@tums.ac.ir Similar research interests

IMPROVEMENT OF REDOX STATE AND FUNCTIONS OF IMMUNE CELLS AS WELL AS OF BEHAVIORAL REPONSE IN OLD MICE AFTER TWO WEEKS SUPPLEMENTATION OF FERMENTED MILK WITH PROBIOTICS

Caroline Hunsche[1], Julia Cruces[1], Mónica De la Fuente[1]

[1]Department of Genetics, Physiology and Microbiology (Unit of Animal Physiology). Faculty of Biology. Complutense University of Madrid.

Research Institute of Hospital 12 de Octubre (i+12) Madrid.

***Corresponding author:**

Mónica De la Fuente

Department of Genetics, Physiology and Microbiology. Faculty of Biology Complutense University of Madrid.

José Antonio Novais Street, 2. 28040. Madrid. Spain.

Telephone number: 0034 91 3944989

Fax number: 0034 91 3944935

Email address: mondelaf@bio.ucm.es

Acknowledgements

This work was supported by grants from the Research Group of Madrid Complutense University (910379ENEROINN) and FIS (PI15/01787) of Institute de Salud Carlos III – Fondo Europeo de Desarrollo Regional (ISCIII-FEDER).

C. H. is the recipient of a PhD fellowship from CNP-q-Brazil.

Compliance with ethical standards

The experiments procedures and handling of animals were performed with approval of the Committee for Animal Experimentation of the Complutense University of Madrid (ref. CEA-UCM 06/2012) and were conducted in accordance with the guidelines and protocols of the Royal Decree 53/2013 regarding the care and use of laboratory animals.

Conflict of interest

The authors declare no conflict of interest.

IMPROVEMENT OF REDOX STATE AND FUNCTIONS OF IMMUNE CELLS AS WELL AS OF BEHAVIORAL REPONSE IN OLD MICE AFTER TWO WEEKS SUPPLEMENTATION OF FERMENTED MILK WITH PROBIOTICS

Abstract

The homeostatic systems, such as the nervous and immune systems, show deterioration in aging as consequence of the age-related oxidative-inflammatory stress establishment. The supplementation with fermented milk containing probiotic bacteria could be a good nutritional strategy to improve homeostatic system functions in aged individuals through the modulation of their redox state. The aim of the present study was to evaluate the effect of two weeks supplementation with a commercial fermented milk containing yogurt probiotics (*Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophiles*), and the probiotic *Lactobacillus casei* DN-114001 on behavior, redox state and immune cell functions of old mice as well as on their life span. Old female ICR-CD1 mice were supplemented with fermented milk containing these probiotics for two weeks. After this period, a variety of behavioral tests were performed and several parameters of redox state and function of peritoneal leukocytes were analyzed. The results showed that the two weeks supplementation of fermented milk containing probiotics improved behavior (such as muscular vigor, exploratory activity and anxiety-like behavior) as well as the redox state and functions of peritoneal immune cells in old mice. In conclusion, the present study shows that the supplementation with fermented milk containing probiotics for a short period of time could be a good nutritional strategy to promote healthy aging.

Keywords: fermented milk with probiotics; behavior; redox state; immune cell functions; old mice.

1. Introduction

The age-related impairment of the homeostatic systems such as the nervous and immune systems, which resulting in an increase of morbidity and mortality, can be caused principally by the oxidative stress (unbalance between oxidants and antioxidants with increase of the first) that appears with aging [9,10]. In fact, according to the oxidative-inflammatory theory of aging, the chronic oxidative stress and the inflammatory stress situations are the basis of the age-related impairment of functions of the organism, the homeostatic system being the most affected. In addition, this theory also suggests that the immune system, due to its property of producing oxidants, which in low levels are essential to carry out an effective immune response, if not well regulated, could be involved in the rate of aging of each individual through the generation of oxidative stress [5,9,10]. Moreover, one of the causes of the age-related changes in the immune system, which is denominated immunosenescence, could be the altered redox state of the immune cells [9,10]. Importantly, the oxidative stress observed in peripheral immune cells with aging seems to reflect the redox state of the rest of the organism [10].

Immunosenescence is characterized by a marked impairment of innate and adaptive immune responses, which includes lower migration to the infectious focus or chemotaxis, phagocytosis and proliferation of immune cells in response to mitogens, as well as lower anti-tumor activity of natural killer (NK) cells [35]. With respect to the nervous system, there is an age-related progressive deterioration of a variety of brain and behavioral functions, including memory, cognitive, and motor activity [6,41,51]. Given that the nervous system and the immune system share a bidirectional communication [9], it is expected that immunosenescence would have an impact on brain function and behavior. In fact, increasing evidence suggests that aged individuals show an increased infiltration of immune cells and oxidative and inflammatory compounds into the brain. This condition is associated with dysfunctions in the brain and increased susceptibility to neurological disorders [13,17].

The adequate function of the homeostatic systems, and consequently the maintenance of health, depends mostly on lifestyle factors, while genetic background seems to influence less [10,26,40]. Thus, several lifestyle strategies have been used to slow down the rate of aging [11]. In this context, nutrition has been proposed as a positive intervention capable of modifying the age-related impairments and concretely the consumption of diet with adequate amounts of antioxidant compounds showed to ameliorate the age-related alterations of the immune system in rodent models of chronological and premature aging, improving redox state and functions of immune cells [11,24]. In addition, increasing evidence suggests that the consumption of food containing probiotic bacteria (such as lactobacilli and bifidobacteria) is a good nutritional strategy to improve health status during the aging process [31]. In this sense, a great body of evidence indicates that the consumption of certain strains of probiotics can improve the immune response of the host [12,15,34,46,48,52,55], as well as brain functions and behavior [7,8,53]. This capacity of probiotics to improve

homeostatic system functions could be carried out, at least in part, through protecting the host against oxidative stress [25,26]. In fact, some studies have shown that certain strains of probiotics exhibit antioxidant properties [22,39].

In particular, the consumption of fermented milk containing yogurt cultures and *Lactobacillus casei* DN-114001 has shown immunological and gastrointestinal benefits in the elderly population. Thus, there is evidence that this dietary supplementation is capable of reducing the duration of infection diseases [21,52] and preventing antibiotic-associated diarrhea in old individuals [14]. In addition, we previously demonstrated that a short-term (one week) supplementation with this same fermented milk drink was able to improve behavior parameters and functions of peritoneal leukocytes in old mice. Furthermore, a longer-term supplementation (four weeks) maintained or loss these improvements in the same immune functions [23]. However, it remains unknown whether the supplementation with this fermented milk drink could affect the redox state of the immune cells. Therefore, we hypothesized that this supplementation for the period of two weeks would ameliorate age-related alterations in the functions of the nervous system (behavior) and immune system (redox state and functions of peritoneal leukocytes). To examine this hypothesis, we investigated the effects of the dietary supplementation with fermented milk containing yogurt cultures and the probiotic *Lactobacillus casei* DN-114001, for two weeks, on several parameters of redox state and functions of peritoneal leukocytes as well as on behavior responses, in old mice. In addition, we analyzed if this supplementation could improve the life span of these animals.

2. Materials and methods

2.1 Animals

Old (72±4 weeks of age) female ICR/CD1 mice (*Mus musculus*) (Janvier SAS, Chassal, France) were used. The animals were housed in polyurethane cages and maintained under standard laboratory conditions (12:12 h reversed light/dark cycle; lights on at 8:00 pm, relative humidity of 50-60%, temperature of 22±2ºC and adequate ventilation). All mice were fed a standard maintenance diet (Teklad Global 14% Protein Rodent Maintenance Diet, Harlan Interfauna Iberica, Barcelona, Spain) and water *at libitum*.

2.2 Experimental groups

Old mice were separated according to their body weight into two groups (n=8-10, per group), namely (1) old control mice which were not dietary supplemented and (2) old mice supplemented with fermented milk containing probiotics for two weeks. The commercial fermented milk drink studied contains two bacterial strains commonly used in yogurt, *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*, and the probiotic bacteria *Lactobacillus casei* DN114001 (10^9 CFU/100g). Weight, food and supplementation intakes were measured every day in the study. After the period of two weeks of dietary supplementation with ferment milk, a variety of tests and assays were performed in old mice in order to evaluate behavior as well as the redox state and functions of peritoneal leukocytes.

2.3 Behavioral tests

Behavioral tests were conducted between 8 am and 10 am during two consecutive days, under illumination of fluorescent lamps (20 W), which followed a procedure previously described [4]. On the first day, a battery of tests (T-maze, wood rod, corner and tightrope tests) was performed in mice. On the second day, mice were subjected to the holeboard test. The experimental apparatus was cleaned before the performance of each mouse in order to avoid olfactory trails.

2.3.1 Motor coordination and equilibrium abilities

In order to assess motor coordination and equilibrium, animals were placed in the center of a wood elevated rod (rod dimensions: 22 cm height, 80 cm length, 2.9 cm width divided in segments of 10 cm) for one trial of 60 s, following a procedure previously described [4].

2.3.2 Muscular vigor and traction

As an index of muscular vigor, mice were hanged by its forelimbs in the middle of an elevated horizontal tightrope (40 cm height, 60 cm length and divided into 6 segments of 10 cm), following a procedure previously described [4].

2.3.3 Vertical exploratory activity

The vertical exploratory activity (studied as the performance of "rearing", that is, when the mouse stands up on his hindlimbs so that his body becomes more perpendicular to the ground) was evaluated in the holeboard test. The holeboard test consists in placing the animal in a box made of wood (60 X 60 X 45 cm) with matte-painted metallic walls, divided into 36 squares (10 X 10 cm), bearing four equally spaced holes (3.8 cm of diameter). All but 20 peripheral squares were considered central. The test was performed during 5 minutes [4].

2.3.4 Horizontal exploratory activity

Horizontal exploratory activity was assessed in the corner test, as the number of corners of the cage visited, and as the percentages of mice that performed peripheral ambulation (number of line crossings in the peripheral area, by the walls) and central ambulation (number of line crossings in the central area) in the holeboard test. Peripheral ambulation was considered lower exploratory activity than central ambulation due to the tendency of mice to remain close to walls (thigmotaxis) [49]. The corner test consists in placing the mouse in a new cage (22.0 X 22.0 X 14.5 cm) with bedding during 30 s. In addition, horizontal exploratory behavior was recorded using the T-maze test, which is a wooden apparatus with three enclosed arms (short arm: 27 X 10 cm; long arm: 64 X 10 cm; walls: 19 cm high) [35]. The mouse was placed inside the "short" arm of the maze with its head facing the wall. The time (in seconds) to complete the test (exploration of all three maze arms) was assessed.

2.3.5 Goal-directed exploratory behavior

The goal-directed exploratory behavior of mice was assessed in the holeboard test. An object formed by a plastic was placed into each of the four holes of the holeboard to attract mice attention. The frequency (percentage and number) of mice that performed head-dips (when the animal places its head into the hole) was considered as a goal-directed exploratory behavior.

2.3.6 Anxiety-like behavior

Repeated self-grooming and digging, and defecation presence were considered as anxiety-like behaviors [3,42]. Thus, the anxiety-like behavior was measured as the percentages of mice that performed repeated self-grooming and digging in the corner test. In addition, the presence of defecation in the T-maze and holeboard tests was also evaluated.

2.4 Collection of peritoneal leukocytes

The peritoneal suspensions were obtained between 8 am and 10 am to minimize circadian variations in the immune system, by a procedure previously described, without animal sacrifice [19,20], which allowed monitoring the life span of the mice. Briefly, 3 ml of Hank´s solution, adjusted to pH 7.4, were injected into the peritoneum, the abdomen was massaged and the peritoneal exudate cells were collected allowing the recovery of 90-95% of the injected volume. The peritoneal leukocytes, consisting of lymphocytes and macrophages, were counted in Neubauer chambers (Blau Brand, Germany). The suspensions were adjusted to a final concentration of $5X10^5$ macrophages or lymphocytes/ml in Hank´s solution or 10^6 leukocytes/ml in Hank´s solution or complete medium (Roswell Park Memorial Institute (RPMI) 1640 enriched with L-glutamine (PAA, Pasching, Austria) and supplemented with 10% heat-inactivated (56ºC, 30 min) fetal calf serum (PAA) and gentamicin (100 mg/ml, PAA) with or without phenol red, depending on the type of assay used. Macrophages and lymphocytes were identified by their morphology. The cellular viability was measured using the trypan-blue (Sigma-Aldrich, Madrid, Spain) exclusion test and in all cases was higher than 98%.

2.5 Immune functions

2.5.1 Chemotaxis assay

Chemotaxis of peritoneal leukocytes was evaluated according to a slight modification of Boyden´s method [19], consisting basically of the use of chambers with two compartments separated by a filter with a pore diameter of 3 µm (Millipore, Bedford, MA, USA). The chemotaxis index (CI) was determined by counting, in an optical microscope (100X), the total number of macrophages and lymphocytes on one third of the lower face of the filters.

2.5.2 Phagocytosis assay

Phagocytosis assay of inert particles (latex beads) was carried out following a method previously described [19]. The number of particles ingested by 100 macrophages was counted using an optical microscope (100X) and

expressed as phagocytic index (PI). The percentage of macrophages, which phagocyted at least one latex bead, was also determined and expressed as phagocytic efficiency (PE).

2.5.3 Natural Killer assay

An enzymatic colorimetric assay was carried out to measure the cytolysis of tumor cells (Cytotox 96 TM Promega, Boehringer Ingelheim, Germany) based on the determination of lactate dehydrogenase enzyme (LDH), as previously described [19]. The results were expressed as percentage of lysis of target cells.

2.5.4 Lymphoproliferation assay

Following the method previously described [20], aliquots (200 µl) of peritoneal leukocytes (10^6 lymphocytes/ml complete medium) were seeded in 96 well flat-bottomed microtitre plates (Numc, Roskilde, Denmark) and 20 µl of concanavaline A (ConA 1µg/ml; Sigma-Aldrich), 20 µl of lipopolysaccharide (LPS, *E. coli*, 055:B5 1µg/ml; Sigma-Aldrich) or 20 µl of complete medium (spontaneous proliferation) were added per well. The cells were harvested in a semiautomatic microharvester 24h later. Thymidine uptake was measured using a beta counter (LKB, Uppsala, Sweden). The results were expressed as ^{3}H-thymidine uptake (cpm).

2.6 Oxidative stress parameters

2.6.1 Catalase activity assay

The activity of catalase (CAT) was determined following a previously described method [1]. The enzymatic assay was followed using spectrophotometry for 80 seconds at 240 nm. The results were expressed as international units (U) of enzymatic activity per 10^6 cells.

2.6.2 Glutathione reductase activity

The glutathione reductase (GR) activity was measured by a method previously described [1]. The total activity was measured through the oxidation of nicotinamide adenine dinucleotide phosphate (NADPH) spectrophotometrically at 340 nm. The results were expressed as international milliunits (mU) of enzymatic activity per 10^6 cells.

2.6.3 Glutathione concentrations

Both reduced (GSH) and oxidized (GSSG) concentrations of glutathione were measured using a fluorometric method, with some modifications [18] Fluorescence was determined in a plate reader (Fluostar Optima, BMG Labtech, Spain) using excitation at 350 nm and emission detection at 420 nm. The results were expressed as nmol/10^6 cells. The GSSG/GSH ratios were then calculated for each sample.

2.7 Statistical analysis

SPSS 21.0 (SPSS, Inc., Chicago, USA) was used for the statistical analysis of the results. The data were expressed as mean ± standard error of the mean (SEM). Each value is the mean of the data from an assay performed in duplicate or triplicate. Normality of the samples was checked by the Kolmogorov-Smirnov test and homogeneity of

variances with the Levene test. The data were statistically evaluated by the Student´s *t*-test for independent and paired samples. $P<0.05$ was considered statistically significant.

3. Results

3.1 Body weight, food and supplementation intakes

At the end of the two weeks supplementation period, there were significant differences in the body weight between old mice supplemented with fermented milk (probiotics) and non-supplemented mice ($P<0.001$. Figure 1). However, no significant differences were observed in the average daily food intake (5 ± 0.2 g/day/mouse, in old mice supplemented with fermented milk and 5 ± 0.2 g/day/mouse, in old non-supplemented mice) and in the average daily water intake (4 ± 0.2 ml/day/mouse, in old mice supplemented with fermented milk and 5 ± 0.3 ml/day/mouse, in old non-supplemented mice). The average daily supplementation of fermented milk intake during the experiment period was 4 ± 1.5 ml/day/mouse.

3.2 Behavioral tests

Table 1 shows the results obtained in the behavioral assessments of old non-supplemented mice and old mice supplemented with fermented milk (probiotics).

In the wood rod test, no significant differences were observed in the parameters that evaluated motor coordination and equilibrium abilities between old mice supplemented with fermented milk and non-supplemented mice (Table 1).

In the tightrope test, old mice supplemented with fermented milk (probiotics) showed significantly lower percentage of mice that fell off from the rope than non-supplemented mice ($P<0.001$. Table 1). However, the supplementation with fermented milk did not result in significant differences regarding the percentage of old mice that completed the test in comparison with old controls (Table 1).

The vertical exploratory activity of mice, which was assessed by the frequency of rearings, is shown in Table 1. The percentage of old mice supplemented with fermented milk that performed rearings in the holeboard test was significantly higher in comparison with non-supplemented controls ($P<0.001$. Table 1). In addition, in the holeboard test, the total number of rearings was significantly higher in old mice supplemented with fermented milk than in old controls ($P<0.01$. Table 1).

The results of horizontal exploratory activity are shown in Table 1. In the corner test, no significant differences were observed in the number of corners visited between old mice supplemented with fermented milk and non-supplemented controls (Table 1). The percentage of peripheral ambulation in the holeboard test was significantly lower in old mice supplemented with fermented milk in comparison with non-supplemented controls ($P<0.05$. Table 1). In contrast, the percentage of central ambulation in the holeboard was significantly higher in old mice supplemented with fermented milk than in non-supplemented controls ($P<0.05$. Table 1). With regards to the T-maze test, old mice

supplemented with fermented milk exhibited lower time (in seconds) to explore the three arms of T-maze than non-supplemented mice (P<0.05. Table 1).

No significant differences were observed in the goal-directed exploratory activity between old mice supplemented with fermented milk and non-supplemented mice (Table 1).

The results of the anxiety-like behavior of mice are shown in the table 1. The supplementation with fermented milk resulted in a higher percentage of old mice performing self-grooming and in a lower percentage of old mice performing repeated digging in the corner test in comparison with non-supplemented mice (P<0.01 and P<0.001, respectively. Table 1). In addition, the presence of defecation in the T-maze and holeboard tests was significantly lower in old mice supplemented with fermented milk than in old non-supplemented controls (P<0.05 and P<0.01, respectively. Table 1).

3.3 Immune function parameters

The peritoneal macrophage functions are shown in Figure 2. The chemotaxis index of macrophages in response to a chemotactic gradient (formylated peptide), which mimics the migration of immune cells towards the infection site, was significantly higher in old mice supplemented with fermented milk (probiotics) in comparison with old non-supplemented mice (P<0.05. Figure 2A). In addition, the number of latex beads ingested by macrophages, which is measured by the phagocytic index (Figure 2B), was significantly higher in old mice supplemented with fermented milk than old non-supplemented controls (P<0.01). Also, the number of macrophages with phagocytic ability, which is measured by the phagocytic efficiency, was significantly higher in old mice supplemented with fermented milk than in old non-supplemented mice (P<0.001. Figure 2C).

The lymphocyte functions are displayed in Figure 3. The chemotaxis index of peritoneal lymphocytes induced by a chemotactic peptide (Figure 3A) was significantly higher in old mice supplemented with fermented milk than in old non-supplemented mice (P<0.05).

The NK cell activity against tumor cells (Figure 3B) was significantly higher in old mice supplemented with fermented milk in comparison with old non-supplemented mice (P<0.05).

The lymphoproliferation in response to T-cell mitogen (ConA) and to B-cell mitogen (LPS) (Figure 3C and 3D) was significantly higher in old mice supplemented with fermented milk with respect to old non-supplemented mice (P<0.05).

3.4 Peritoneal leukocyte oxidative stress parameters

The results of enzymatic and non-enzymatic antioxidants, such as catalase and glutathione reductase activities, as well as reduced glutathione (GSH), respectively, are shown in Figure 4. The activity of the antioxidant enzymes catalase and glutathione reductase was significantly higher in old mice supplemented with fermented milk (probiotics) than in non-supplemented controls (P<0.05. Figures 4A and 4B). However, no significant differences were observed in

the concentration of GSH between old mice supplemented with fermented milk and non-supplemented controls (Figure 4C).

The results of the values of non-enzymatic oxidants, which includes the concentration of oxidized glutathione (GSSG) and the GSSG/GSH ratios are shown in Figure 5. Old mice supplemented with fermented milk (probiotics) exhibited lower GSSG concentrations than old non-supplemented mice ($P<0.05$. Figure 5A). However, no significant differences were found in the GSSG/GSH ratios between old mice supplemented with fermented milk and non-supplemented mice (Figure 5B).

3.5 Life span

The differences were not statistically significant in the life span of mice after the 2 weeks of supplementation with fermented milk. The average of life span of mice supplemented with fermented milk containing probiotics was 122 ± 5 weeks, whereas in non-supplemented mice the average of life span was 118 ± 6 weeks old.

4. Discussion

Although we previously demonstrated that the short-term (one week) supplementation with fermented milk containing yogurt cultures plus *Lactobacillus casei* DN-114001 was able to improve behavior parameters and functions of immune cells in old mice [23], the effect of this supplementation on redox state parameters has not been studied. In the present work, the changes in several parameters of oxidative stress have been studied in peritoneal leukocytes, together with functions of these cells and behavior response in old mice after the supplementation with this same fermented milk drink during a different period of time (two weeks). In addition, we analyzed the body weight of theses animals.

The results of the present study found that the supplementation with fermented milk containing probiotics during two weeks was able to improve behavior as well as the redox state and functions of peritoneal leukocytes in old mice. Moreover, during the supplementation period, there were significant differences in the body weight between old mice supplemented with fermented milk (probiotics) and non-supplemented mice. Thus, old supplemented mice maintained a similar body weight during this period of two weeks, whereas old non-supplemented mice had a significant loss of body weight. In this sense, it is known that the maintenance of a constant body weight during advanced aging is considered a positive health outcome, since the age-related body weight loss is often associated with malnutrition and sarcopenia, as well as is one of the parameter characteristic of frailty [25,29,35]. In fact, malnutrition, sarcopenia and frailty have been associated with increased risk of morbidity and mortality in older age [25,29,35]. Nevertheless, the consumption of fermented milk with probiotics did not result in significant differences in the life span of mice, similarly to that observed when mice were supplemented with this fermented milk drink for the period of four weeks [23]. Thus, it is possible that the supplementation with fermented milk at old age is not sufficient to extend the life span of mice, although the benefits found on behavior, redox state and immune functions could indicate a healthier

aging. In fact, the immune cell function parameters studied here have been shown to predict health status [35]. Another strategy of lifestyle, such as the environment enrichment, has also shown an improvement of the redox state and immune functions in old mice without a significant effect on longevity. However, when this strategy was applied from adult age, there was a significant increase of the life span [2].

With aging, there is a progressive deterioration of brain and behavioral functions, such as muscular vigor, motor coordination, and equilibrium [44,45]. In addition, old individuals are more likely to suffer from mental disorders, such as anxiety [16]. In the present study, the supplementation with fermented milk during two weeks was able to restore this impaired behavioral response of old mice. Thus, several behavioral parameters, such as muscular vigor (assessed by the tightrope test), vertical exploratory activity (assessed by the performance of rearings) and horizontal exploratory activity (assessed by the ambulation of mice in the holeboard test) were improved in old mice supplemented with fermented milk. In addition, these supplemented animals showed a higher central exploratory activity in the holeboard. In this sense, previous studies indicate that the central ambulation is associated with lower anxiety-like behavior, given that mice prefer to stay in the peripheral area (close to the walls), avoiding the central area (which is considered more threatening for mice) [49,50]. Moreover, the behavior of repeated digging and the presence of defecation, which may be considered anxiety-related measures [3,42], were significantly lower in old mice supplemented with fermented milk than in non-supplemented mice. Nevertheless, the performance of self-grooming, which is usually considered an anxiety-like behavior [27], was higher in old supplemented animals than in non-supplemented controls. In this regard, previous studies suggest that the self-grooming behavior could be elicited by both opposite conditions, such as comfort and stress [28]. Thus, it is possible that the higher performance of self-grooming in old supplemented mice could indicate comfortable, rather than stressful situations. These results are in agreement with those previously found in old mice supplemented with the same fermented milk during one week [23]. Thus, a longer time of supplementation (two weeks) maintained these improvements. In addition, other studies performed in different probiotic strains (*Lactobacillus plantarum*, *L. fermentum*, *L. rhamnosus*, *L. helveticus* R0052, *Bifidobacterium longum* 1714, *B. breve* 1205 and *B. longum* R0175) have also shown their positive effects on behavior, including improved motor coordination and reduced anxiety-like behavior, in young and adult rodents [7,53,37,43]. The mechanism by which probiotics affect brain function and behavior remain unclear, but some evidence suggests the association of ingestion of probiotics with microbial composition changes, immune activation and production of neurometabolites [8].

Antioxidant defenses are an important mechanism developed by organisms against reactive oxygen species (ROS)-induced oxidative stress. In this study, old mice supplemented with fermented milk displayed higher activity of the antioxidant enzyme catalase. Catalase acts eliminating the excess of hydrogen peroxide (H_2O_2). In addition, the non-enzymatic antioxidant tripeptide glutathione (GSH), which is described as the major thiol intracellular redox buffer in cells, is known to exist in its reduced (GSH) or oxidized (glutathione disulphide or GGSG) forms. GSSG is reduced

back to GSH by the action of glutathione reductase (GR) [36]. In the present study, we found a higher activity of GR as well as a lower concentration of oxidized glutathione (GSSG). These results are indicative of lower oxidative stress in peritoneal leukocytes of old mice supplemented with fermented milk. In this sense, increasing evidence suggests that certain strains of lactobacilli and bifidobacteria have antioxidant properties both *in vivo* and *in vitro*, and therefore could decrease oxidative stress [30,32,38,47,50,56]. In particular, a previous study showed that the supplementation of fermented milk with *Lactobacillus fermentum* for two months enhanced the activity of several antioxidant enzymes (superoxide dismutase, catalase and glutathione peroxidase) in old mice [46]. The mechanisms of the antioxidant action of probiotics are largely unknown, however, some evidence suggests that probiotics may modulate the redox status of the host via their metal ion chelating ability, ROS scavenging, enzyme inhibition, and regulating signaling pathways [54].

With respect to the functions of immune cells, the present supplementation was also able to improve age-related impairments of macrophage functions [10,35]. Thus, these supplemented animals displayed enhanced migration (chemotaxis) and phagocytic capacity of peritoneal macrophages in comparison with non-supplemented mice. In agreement, our previous results also showed immune-enhancing proprieties after the consumption of the same fermented milk drink during one week [23]. Another study, which also used the same fermented milk drink, demonstrated that this supplementation, in young mice, resulted in increased phagocytic activity of peritoneal macrophages [12]. In addition, the supplementation with the probiotic *Bifidobacterium lactis* Bi-07 or *Lactobacillus fermentum*, in aged humans and mice, respectively, was able to enhance the phagocytosis of monocytes and neutrophils [34,46].

With aging, NK cells show lower anti-tumor activity [35]. However, the supplementation with fermented milk during two weeks was able to increase this immune function in old supplemented mice in comparison with non-supplemented mice. In agreement, our previous results showed increased anti-tumor activity of NK cells after one and four weeks of supplementation [23]. Similarly, the ingestion of a commercial fermented drink containing *Lactobacillus casei* Shirota for four weeks increased the NK activity of healthy aged people [15].

The functions carried out by lymphocytes, such as migration and proliferation in response to T cell-specific mitogen (ConA) or B cell-specific mitogen (LPS), suffer age-related impairments [35]. The supplementation with fermented milk, however, was able to improve these functions in old mice. Although these results are in agreement with those found in the long-term supplementation (four weeks) as we observed in our previous work, the short-term supplementation (one week) was not able to improve the mitogen-stimulated lymphocyte proliferation [23]. Thus, it seems that this immune response of lymphocytes, which is considered a typical function of the adaptive immunity, are only improved after a period of supplementation of at least two weeks.

In conclusion, the present study shows that the supplementation of a commercial drink containing probiotics during two weeks improved behavior (such as muscular vigor, exploratory activity and anxiety-like behavior), as well as the redox state and functions of peritoneal leukocytes in old mice. Given that oxidative stress is the main contributor of age-related impairments in the homeostatic systems (including the nervous and immune systems), the supplementation of fermented milk with probiotics could be a good nutritional strategy to ameliorate these impairments in old individuals through the modulation of their redox state. Moreover, since the parameters of immune function analyzed are markers of health [35], and it has been recently demonstrated that the age-related alterations in these functions are similar in mice (peritoneal leukocytes) and in humans (blood leukocytes) [10,35], we could suggest the use of this strategy to improve health in the elderly.

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Table 1. Behavioral tests in old mice supplemented with fermented milk (probiotics) and old non-supplemented mice.

	OLD MICE	
	CRTL	FERMENTED MILK WITH PROBIOTICS
Motor coordination and equilibrium (Wood rod test)		
% of mice falling off	19	9
Total number of crossings segments	4±0.5	5±1
% of mice that complete the test	60	73
Muscular vigor (Tightrope test)		
% of mice falling off	90	55***
% of mice that complete test	10	18
Traction (Tightrope test)		
% of mice that show maximum traction capacity	50	45
Non-goal-directed exploratory behavior		
Vertical exploratory activity		
% of mice that perform rearing (Holeboard test)	50	91***
Total number of rearings (Holeborad test)	25±3	38±4**
Horizontal exploratory activity		
Number of corners visited (Corner test)	4±2	4±3
Total number of line crossings (Holeboard test)	316±22	304±15
% of mice that perform central ambulation	35±2	47±2*
% of mice that perform peripheral ambulation	65±2	53±2*
Time to complete the test (s) (T-maze test)	25±4	16±1*
Goal-directed exploratory activity		
% of mice that perform head-dips (Holeboard test)	100	100
Number of head-dips (Holeboard test)	8±1	8±1
Anxiety-like behavior		
% of mice that perform self-grooming (Corner test)	0	9**
% of mice that show repeated digging (Corner test)	50	9***
% faeces presence (T-maze test)	20	9*
% faeces presence (Holeboard test)	100	91**

Results are expressed as percentage (%) or mean ± *SEM* in old control mice (n=8-10) and old mice supplemented with fermented milk (n=8-10) *P<0.05; **P<0.01; ***P<0.001 with respect to the values of old control mice.

FIGURE LEGENDS

Figure 1. Body weight control in old control mice and old mice supplemented with fermented milk (probiotics) during two weeks. Each group represents the mean ± *SEM* of 8-10 values corresponding to that number of animals *** $P<0.001$; ** $P<0.01$; * $P<0.05$ with respect to the values of old control mice.

Figure 2. Macrophage functions. **(A)** Macrophage chemotaxis index (CI, number of macrophages). **(B)** Macrophage phagocytic index (PI, number latex beads/100 macrophages). **(C)** Macrophage phagocytic efficacy (PE, number of phagocyting macrophages/100 macrophages). Each column represents the mean ± *SEM* of 8-10 values corresponding to that number of animals and each value being the mean of duplicate or triplicate assays. *** $P<0.001$; ** $P<0.01$; * $P<0.05$ with respect to the values of old control mice.

Figure 3. Lymphocyte functions. **(A)** Lymphocyte CI (number of lymphocytes). **(B)** Natural killer cell activity (% lysis). **(C)** Lymphoproliferative response to concanalvaline A (ConA) (cpm). **(D)** Lymphoproliferative response to lipopolysaccharide (LPS) (cpm). Each column represents the mean ± *SEM* of 8-10 values corresponding to that number of animals and each value being the mean of duplicate or triplicate assays. *** $P<0.001$; ** $P<0.01$; *$P<0.05$ with respect to the values of old control mice.

Figure 4. Antioxidant parameters. **(A)** Catalase activity ($U/10^6$ cells). **(B)** Glutathione reductase ($mU/10^6$ cells). **(C)** Reduced glutathione concentration ($nmol/10^6$ cells). Each column represents the mean ± *SEM* of 8-10 values corresponding to that number of animals and each value being the mean of duplicate or triplicate assays. *$P<0.05$ with respect to the values of old control mice.

Figure 5. Oxidative parameters. **(A)** Oxidized glutathione concentration ($nmol/10^6$ cells). **(B)** Ratio Oxidized glutathione (GSSG)/reduced glutathione (GSH). Each column represents the mean ± *SEM* of 8-10 values corresponding to that number of animals and each value being the mean of duplicate or triplicate assays. *$P<0.05$ with respect to the values of old control mice.

Figure 1

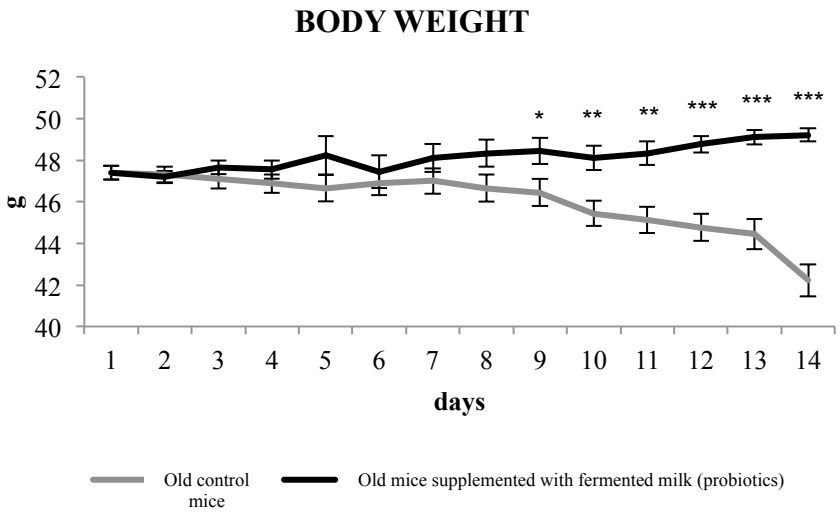

Figure 2

Click here to access/download;Figure;Figure 2 macrophage functions.pdf

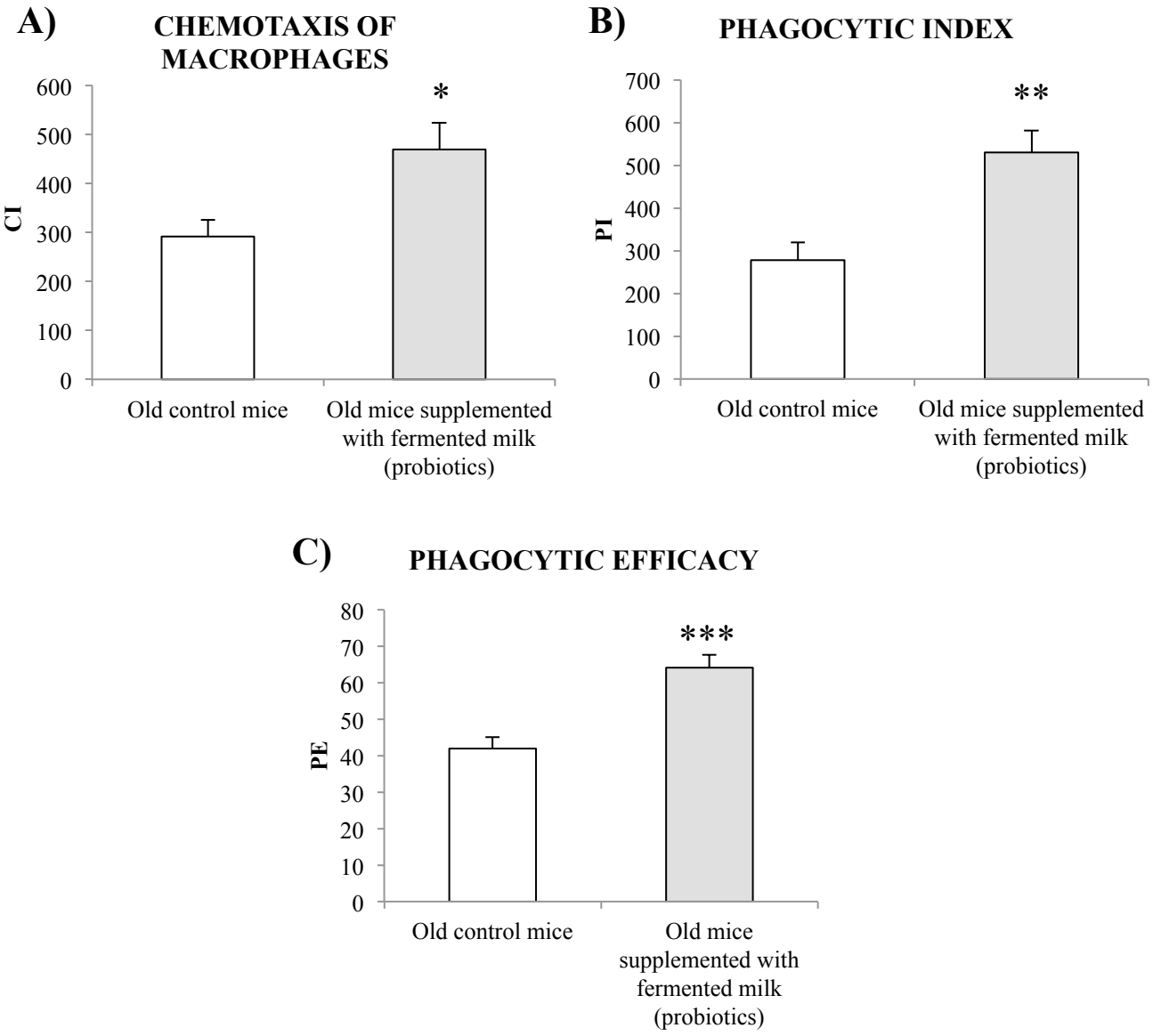

Figure 3

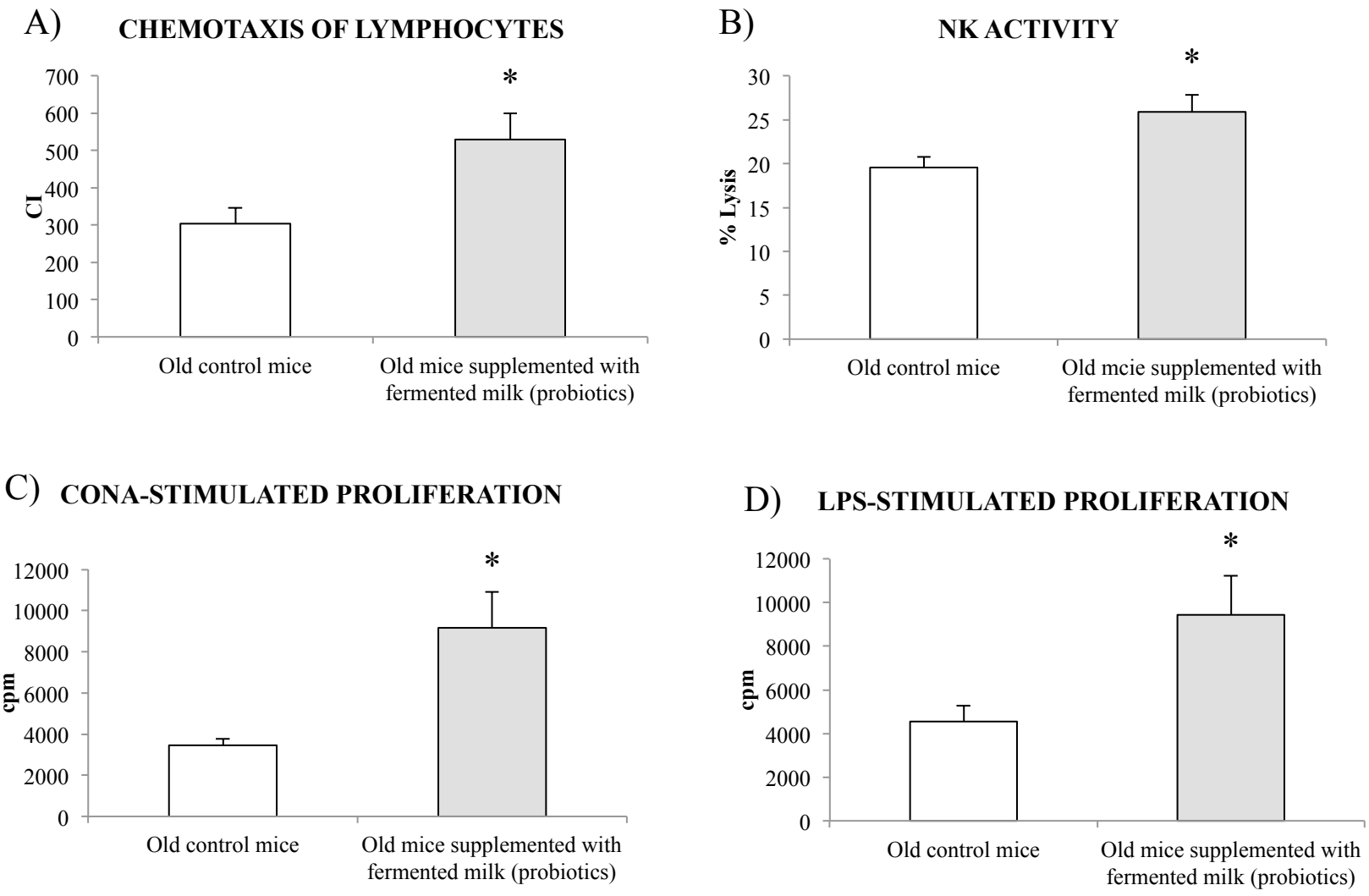

Figure 4

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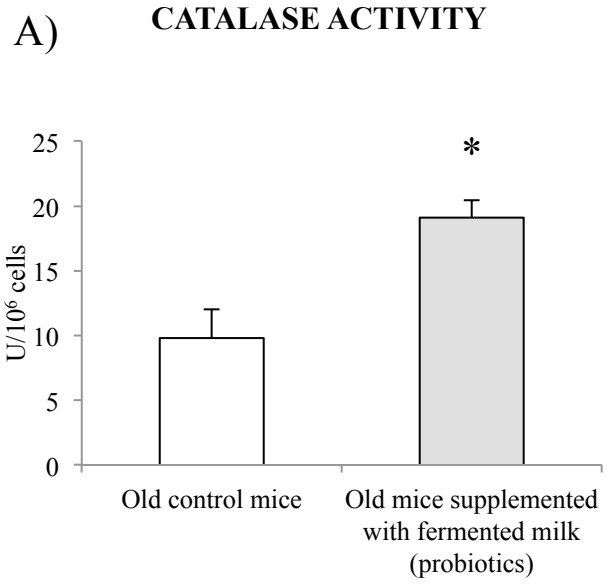
A)



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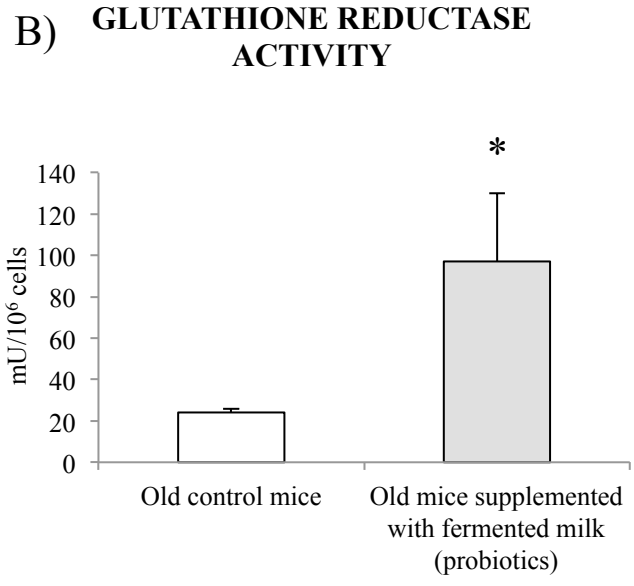

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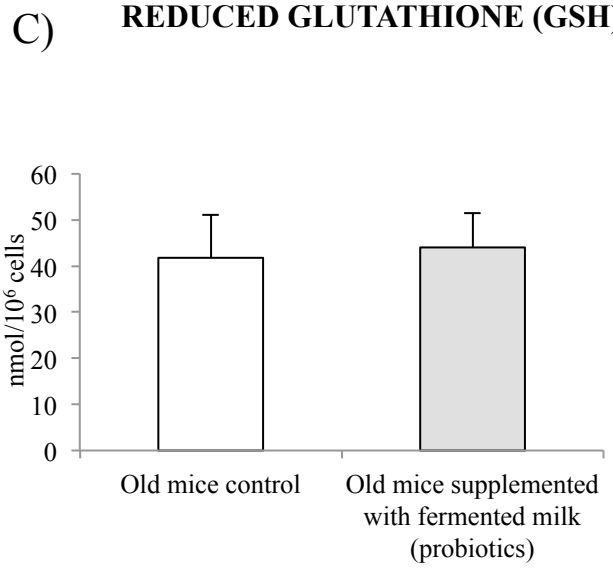

Figure 5

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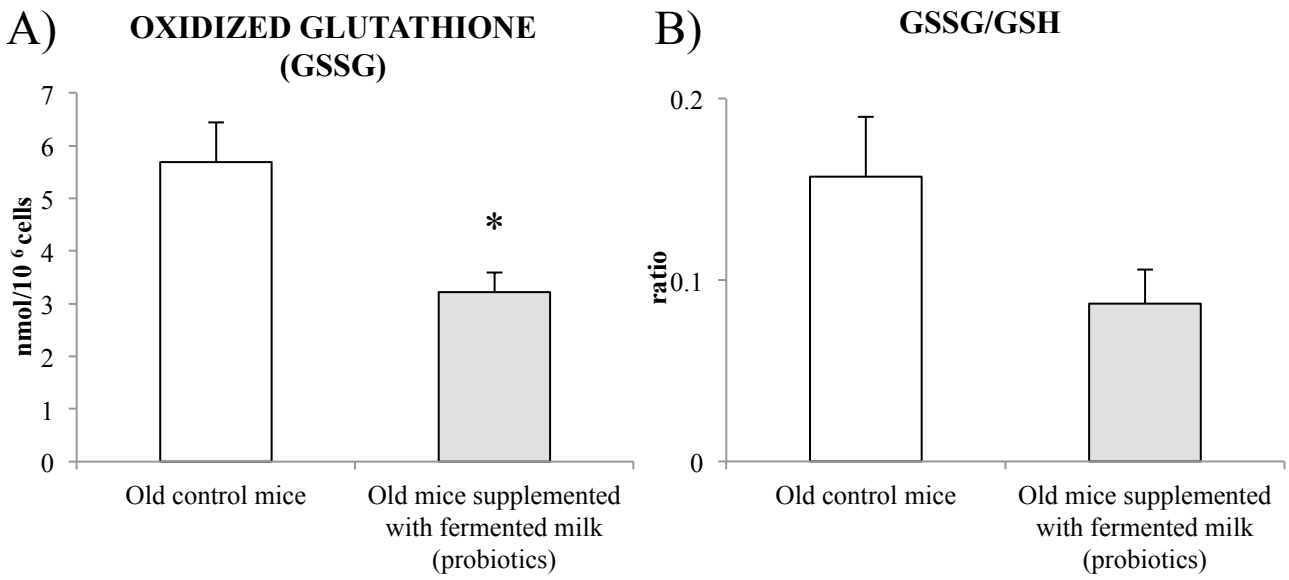

4. DISCUSSION

4.1. DISCUSSION OF RESULTS

4.1.1. Effects of diet-induced obesity onset on behaviour, immune function and redox/inflammatory state at different ages, as well as on the life span of mice

In this thesis we wanted to study as the first sub-objective **the effects of the early adulthood diet-induced obesity onset on immune function and redox/inflammatory state of adult female mice, as well as on their life span.** The results obtained showed that a high fat intake during adolescence produced obesity in adult age associated with premature immunosenescence, and this condition was aggravated throughout aging.

Adolescent female mice progressively and significantly increased their body weights by the administration of a high-fat diet for 14 weeks. These animals also presented significantly elevated concentrations of triglycerides and higher systolic arterial pressures when compared to the control group. Moreover, these mice showed conditions associated with obesity similar to those obtained in mice of a previous study in which an identical experimental design was followed to generate adult obesity (Baccan *et al*., 2013). Thus, the administration of a high-fat diet during adolescence seems to be a good model to induce obesity in adult mice. Additionally, this type of diet and the higher adiposity have also been reported to increase the levels of oxidation and inflammation and to promote a dysfunctional immune response (Bailey-Downs *et al*., 2013; Lamas *et al*., 2004; Swindell *et al*., 2010). Since obesity has been proposed as a possible model of premature immunosenescence (De la Fuente and De Castro, 2012), it seemed relevant to evaluate whether obesity generated at adolescence, a critical development period, could produce some features of immunosenescence at adult age. Moreover, it is also important to know if individuals that started their aging process at adult age with premature immunosenescence reached old age with a higher deterioration of immune response than those adults that start their aging showing an appropriate immune response. In this first sub-objective, we found, on one hand, that the development of obesity during adolescence

promoted a premature immunosenescence and oxidative state of peritoneal leukocytes in early adulthood. Thus, adult diet-induced obese (DIO) mice, when compared to controls of the same age, presented several deteriorated immune parameters, such as chemotaxis of both macrophages and lymphocytes, phagocytosis of macrophages, anti-tumour NK activity, mitogen-stimulated lymphoproliferation and mitogen-stimulated release of several cytokines (IL-1β, TNF-α, IL-6, IL-2 and IL-10) derived from leukocyte culture supernatants. These parameters showed values similar to those of chronologically old animals. On the other hand, these adult DIO mice with a premature immunosenescence reached old age in worsened conditions, although they were fed a standard diet during aging.

With respect to the functions studied in the peritoneal macrophages, which represent the first line of immune response, adult DIO mice showed diminished chemotaxis, phagocytosis as well as stimulated intracellular concentrations of superoxide in comparison with adult non-DIO control mice. These parameters showed similar values to those of old mice. Previous studies confirmed an impaired migration of macrophages to the site of inflammation, a diminished phagocytosis capacity as well as decreased levels of intracellular superoxide needed to kill intracellular pathogens in aged individuals (Arranz *et al*., 2010a; De la Fuente *et al*., 2004a; Linehan *et al*., 2014). Also, studies in obese individuals (both rodents and humans) corroborated these findings, indicating a decreased phagocytic capacity and an impaired oxidative burst of macrophages (Lee *et al*., 1999; Mancuso *et al*., 2002; Morrow *et al*., 1985; Palmblad *et al*., 1980). Nevertheless, another study performed in obese humans showed a significantly increased phagocytosis and oxidative burst activity by monocytes and neutrophils (Nieman *et al*., 1999), which could indicate a compensatory mechanism to possible impaired immune cell functions, as has been previously suggested in another study on obesity (McInnis *et al*., 2014). Moreover, a different assay technique was used in this study, which could also explain the different results obtained.

With respect to the anti-tumour NK activity, also a critical player in innate immunity, the data obtained in the present thesis showed a decreased cytotoxic capacity in old and in adult DIO

mice compared to adult controls. Previous research demonstrated that aged individuals, despite their increased number of NK cells, presented depressed NK cytotoxicity (Arranz *et al*., 2010a; Hazeldine and Lord, 2013). Obesity also seems to produce an impaired NK cell activity. In agreement with our results, earlier findings indicate that DIO rats and mice, fed a cafeteria diet and a high-fat diet, respectively, as well as obese human subjects, suffered from a diminished NK cell cytotoxicity (De la Fuente and Castro, 2012; Lamas *et al*., 2004; Morrow *et al*., 1985; O´Shea *et al*., 2010). Moreover, another study found that DIO mice fed a high-fat diet exhibited a poor cytotoxicity against influenza infection and increased mortality (Smith *et al*., 2010).

In addition, immune functions driven by T and B lymphocytes, such as migration and proliferation, were affected by the early onset of obesity. In fact, the present findings showed that both adult DIO and old mice presented similar deteriorated values in relation to peritoneal lymphocyte functions, including chemotaxis and proliferation in response to T and B cell mitogens such as ConA and LPS, respectively, in comparison with adult controls. In agreement, there is previous evidence showing that aged individuals exhibited both impaired chemotaxis and proliferative response to mitogens of lymphocytes (Arranz *et al*., 2010a; Maijó *et al*., 2014). In relation to obese individuals, we did not find studies that evaluated chemotaxis of lymphocytes. Therefore, our results regarding chemotaxis of lymphocytes seem to be the first to describe a decrease in this activity of DIO mice. In relation to the proliferative response of lymphocytes to mitogen stimulation, several studies indicate a reduced capacity of this parameter in obese individuals. In obese mice, Sato Mito *et al*. (2009) found a decreased splenocyte proliferative response with T and B cell mitogens. Moreover, obese humans also exhibited suppressed stimulated proliferation of T and B lymphocytes (Nieman *et al*., 1999; Tanaka *et al*., 1993).

Increasing evidence suggests that the conditions of aging and obesity are accompanied by overactivation of some innate immune functions with increased production of pro-inflammatory cytokines, such as TNF-α, IL-6 and IL-1β, especially in basal conditions (Alvarez-Rodríguez *et al*., 2012; Arranz *et al*., 2010c; Martin-Cordero *et al*., 2009; Shaw *et al*., 2013; Tilg and Moschen,

2006). However, a previous study showed decreased levels of IL-1β, TNF-α, IL-6, IL-2 and IL-10 in peritoneal leukocytes from aged mice under mitogen-stimulated states (Arranz *et al*., 2010c). The present data agree with these results. Thus, following stimulation with mitogens ConA and LPS, the secretion of these cytokines presented a decrease (tendency or statistically significant differences) in old and in adult DIO mice in comparison with adult controls. These findings are in agreement with those obtained in DIO mice, in which peritoneal macrophages, in response to a bacterial infection, exhibited low levels of the pro-inflammatory cytokines TNF-α, IL-β and IL-6 (Amar *et al*., 2007; Lawrence *et al*., 2012; Martin-Cordero *et al*., 2009). A decrease in IL-6 of spleen cells under LPS-stimulated conditions (Baumgarner *et al*., 2014) and of IL-2 following mitogen stimulation (Mito *et al*., 2000) from obese with respect to lean human individuals have also been shown. Nevertheless, in young-adult overweight human subjects, the generation of IL-2 by mononuclear or lymphocyte cells in response to LPS increased, while the levels of IL-10 were similar between obese and lean individuals (Han *et al*., 2011). One possible explanation for these differences could be that individuals were considered mildly obese and thus might not produce significantly impaired immunity. The present results also demonstrated that the release of mitogen-stimulated IL-2 was further impaired in adult DIO mice with respect to old controls. Among several factors that modulate the release of cytokines, prostaglandin E2 (PGE2) has been described to inhibit the release of IL-2 (Santoli *et al*., 1990). Even though this inflammatory mediator is increased in both processes of aging and obesity (Osborn *et al*., 2008; Wu *et al*., 1998), a study found that adipocytes from young obese mice displayed higher levels of PGE2 with respect to old mice (Wu *et al*., 2013). Therefore, it is possible that the release of IL-2 was more suppressed in adult DIO mice by higher levels of PGE2 than in old controls.

Oxidative stress, which is generated by an increase of oxidants and a decrease of antioxidants, has been linked to aging and obesity (De la Fuente and Miquel, 2009; Kregel *et al*., 2007; Vincent *et al*., 2007). In fact, oxidative stress seems to contribute to the pathogeneses of several diseases that are common to both aging and obesity, including diabetes mellitus,

cardiovascular diseases, and cancer (Fernández-Sánchez *et al*., 2011). In the present results, old and adult DIO mice exhibited elevated xanthine oxidase activity (an enzyme that produces oxidants) and diminished catalase activity and glutathione concentrations (two relevant antioxidant defences). Previous studies confirmed that peritoneal leukocytes from old mice or from prematurely aging mice showed enhanced activity of xanthine oxidase (Vida *et al*., 2011; 2014) and a decrease of enzymatic and non-enzymatic antioxidants, such as catalase activity and glutathione concentration (Arranz *et al*., 2000a; Alvarado *et al*., 2006; De la Fuente and Miquel, 2009). In obese individuals an increased production of oxidants and a decreased capacity of antioxidants have also been observed. Thus, Saiki *et al*. (2001) found increased levels in the blood of serum hypoxanthine and uric acid in obese compared to non-obese individuals, while Chiney *et al*. (2011) found that xanthine oxidase activity was significantly elevated in obese when compared to non-obese children. Regarding antioxidant defences, a decrease of glutathione concentrations in erythrocytes of obese individuals during their childhood and adulthood was reported (Charles *et al*., 2008; Codoñer-Franch *et al*., 2010; Pastore *et al*., 2012). In addition, catalase activity was significantly lower in erythrocytes of obese women than non-obese women (Amirkhizi *et al*., 2014). Therefore, these data indicate that the early adulthood diet-induced obesity onset might produce a similar oxidative stress to those reported in the elderly and this condition could contribute to the premature aging of obese individuals.

Surprisingly, when DIO animals grew older, although they were fed a standard diet during their aging process, they continued to exhibit a significant higher body weight than old controls. Biochemical parameters, such as glucose and triglycerides, as well as systolic blood pressure levels, were also significantly increased in old DIO mice. In addition, these animals, which maintained obesity during their aging process, showed an increased deterioration of the immune and oxidative parameters, with respect to old non-DIO mice. This aggravated immunosenescence was reflected in the life span of mice, although not statistically significant, DIO mice tended to exhibit a shorter life span than non-DIO mice. In addition, the consumption of a standard diet across aging was not able

to restore functions and the redox state of the immune cells to the normal levels of the corresponding chronological age.

In a second sub-objective we wanted to study **the effects of the late adulthood diet-induced obesity onset on behaviour, immune function and redox state parameters of middle-aged male and female mice, as well as on their life span.** The results obtained showed that the late adulthood diet-induced obesity onset led to significant impairments in behaviour as well as in functions and redox state of peritoneal leukocytes of middle-aged male and female mice, with males being significantly more affected than females. Thus, middle-aged male diet-induced obese (DIO) mice exhibited greater impairments in a variety of behavioural, immune function and redox state parameters, and consequently showed a shorter life span than their female counterparts. In the middle-aged female DIO mice some parameters presented values similar to those of old female animals. These data confirm the state of accelerated aging as a consequence of the onset of diet-induced obesity in late adulthood. In addition to obesity-related impairments of the immune system, other factors, such as insulin resistance (Frasca *et al*., 2017), dysbiosis and increased intestinal permeability (Boulangé *et al*., 2016), could also contribute to oxidative and inflammatory systemic stresses, and consequently to the process of accelerated aging in obese individuals.

Previous studies have shown that the high-fat diet intake is a good model to develop obesity in mice (Baccan *et al*., 2013; Kanasaki and Koya, 2011; Lutz and Woods, 2012). In addition, this experimental animal model of obesity mimics human obesity, regarding its etiologic aspects (Kanasaki and Koya, 2011). Thus, this is applied to both sexes, since in the present study, middle-aged male and female mice fed on a high-fat diet for 14 weeks of their late-adulthood displayed significantly higher body weight and fat mass than their respective non-DIO counterparts. Moreover, middle-aged male and female DIO mice showed high concentrations of triglycerides and total cholesterol, which are well-known common features of obesity (Klop *et al*., 2013). Also, the late adulthood diet-induced obesity onset affected in a different manner the total body fat of middle-

aged male and female mice. Thus, female DIO mice showed significantly higher body fat mass in comparison with their male counterparts. In agreement, previous studies found that females have a higher propensity to gain body weight and consequently to store more fat in the adipose tissue than males (Mauvais-Jarvis, 2015). In general, females, prior to menopause, tend to accumulate more fat in the subcutaneous adipose tissue, while males in the visceral adipose tissue. This visceral accumulation of fat has been associated with worsened metabolic outcomes in this sex (Palmer and Clegg, 2015). In fact, in the present study, middle-aged male DIO mice showed higher plasma concentrations of glucose and total cholesterol than their female counterparts.

With respect to the results of behavioural tests, we observed lower motor coordination, equilibrium, muscular vigour, horizontal and vertical exploratory activities as well as higher anxiety in middle-aged male and female DIO mice than their respective non-DIO controls. Middle-aged male and female DIO mice displayed a significantly decreased central ambulation in the holeboard test, which indicates low exploratory activity and high anxiety behaviour, since mice with anxiety tend to ambulate more in the peripheral area (close the walls) (Simon *et al*., 1994). Moreover, middle-aged male and female DIO mice exhibited a longer time to complete the exploration of the three arms of the T-maze test than their respective non-DIO counterparts, which also indicates a lower exploratory activity. Regarding the elevated plus maze test, middle-aged male and female DIO mice displayed a decreased percentage of time in the open arms. It is known that a decreased activity in the open arms of the elevated plus maze apparatus indicates higher anxiety behaviour (Walf and Frye, 2007). Moreover, the behaviour of self-grooming and the presence of defecation, which could be considered as anxiety-related measures (Archer, 1973; Sake and Graybiel, 2003), were also significantly higher in middle-aged male and female DIO mice in comparison with their respective non-DIO controls. Previous studies indicate decreased cognitive and exploratory activity as well as increased anxiety in DIO mice. However, in these studies, the high-fat diet was administered soon after weaning (André *et al*., 2014; Arnold *et al*., 2014; da Costa *et al*., 2015; Sharma and Fulton, 2013; Vallodolid-Acebes *et al*., 2011; Yamada *et al*., 2011). Studies in humans,

including data from young to old individuals, also observed an association between increased body weight and worse performance on motor skills, equilibrium and muscular vigour (Deforche *et al*., 2009; Gentier *et al*., 2013; Kumar *et al*., 2008; Maffiuletti *et al*., 2007). Accordingly, it can be suggested that obese individuals perform worse motor tasks because a greater proportion of excess mass has to be supported or moved against gravity during these tasks. However, the poor performance of fine motor tasks cannot be solely explained by the excess of fat, given that fine motor skills are not directly influenced by mass (Gentier *et al*., 2013). In this sense, obese individuals have shown lower neural efficiency and slower integration and processing of information than non-obese individuals (Gunning-Dixon and Raz, 2000; Mehta and Shortz, 2014). In addition, an association has been found between increased muscular fat infiltration in obese and aged individuals and decreased muscle quality and strength (Marcus *et al*., 2010; Moore *et al*., 2014). The present results also showed differences between males and females in the evaluation of behavioural tests, with males being more affected than females. Thus, middle-aged male DIO mice showed a worse performance than their female counterparts in the tests that evaluated motor coordination, equilibrium, muscular vigour, vertical and horizontal exploratory activities and anxiety-like behaviour. However, most of the previous studies on obesity performed in rodents have used a single sex (André *et al*., 2014; da Costa *et al*., 2015; Sharma and Fulton, 2013; Vallodolid-Acebes *et al*., 2011; Yamada *et al*., 2011), thus there is little information comparing the behavioural effects of obesity in both sexes. Nevertheless, in agreement with our data, a previous study considering sex differences in the performance of behavioural tests, showed that males were more vulnerable than females to high-fat diet (HFD)-induced impairments, such as learning functions and synaptic plasticity (Hwang *et al*., 2010). The mechanism underlying the higher impact of HFD and obesity in males than in females remains unknown, but some evidence indicates that oestrogen could have a positive effect in protecting female mice. In this study, male mice treated with estradiol showed less adiposity than controls (Salinero *et al*., 2018).

With respect to the functions studied in the peritoneal macrophages, middle-aged male and female DIO mice showed diminished chemotactic and phagocytic capacities of these cells in comparison with their respective non-DIO counterparts. These results are in agreement with those obtained in our previous sub-objective in which obesity in early adulthood was induced during the adolescent period. Thus, both the different ages of obesity onset (early and late adulthood), resulted in an impaired innate immune response. In addition, in this sub-objective we also evaluated the immune effects of obesity in males. The results showed that middle-aged male DIO mice had lower chemotactic and phagocytic indexes than their respective female counterparts. Moreover, differences were also observed in non-DIO controls, with males showing lower values than females in some functions. Previous evidence confirmed that males display a lower innate immune response against infection in comparison with females (Jaillon *et al*., 2017). Thus, it is possible that obesity could further exacerbate these aspects of the innate immunity of males.

Regarding the NK anti-tumour activity of peritoneal leukocytes, middle-aged male and female DIO mice showed a lower response than their non-DIO counterparts, although no differences were observed between males and females in this function. The migration capacity of lymphocytes to a chemoattractant was significantly lower in middle-aged male and female DIO mice in comparison with their respective non-DIO controls. Similarly, the proliferation of lymphocytes in ConA and LPS-stimulated conditions, two mitogens for T and B lymphocytes, respectively, was also lower in middle-aged male and female DIO mice than in their non-DIO counterparts. These results are in agreement with those obtained in adult DIO female mice that ingested the fat rich diet during adolescence. Thus, both early and late adulthood obesity onsets resulted in impaired functions of lymphocytes. Although in controls middle-aged males showed lower values than females in chemotaxis of lymphocytes, male DIO mice presented higher chemotactic index and LPS-stimulated proliferation of lymphocytes than their female counterparts. The higher response of these immune functions in males could possibly be interpreted as a

compensatory mechanism to counteract the decreased response found in innate immune cell functions (Franceschi *et al*., 1995), especially in the context of obesity.

Oxidative stress, which is generated by an imbalance between oxidants and antioxidants in favour of oxidants, has been associated with the development of obesity, aging and age-related health complications (Bauer and De la Fuente, 2016; Marseglia *et al*., 2015). In the current results, middle-aged male and female DIO mice showed high values of oxidants, such as xanthine oxidase activity, oxidized glutathione (GSSG) and lipid peroxidation (MDA) concentrations, and GSSH/GSH ratios as well as low values of antioxidant defences (such as catalase activity and GSH concentrations). These results are partly in concordance with our previous sub-objective in which it was found an increased oxidative stress in adult DIO female mice was found. However, on the contrary, the activity of glutathione peroxidase (an antioxidant enzyme) was significantly higher in middle-aged DIO animals in comparison with their respective non-DIO counterparts. In this sense, it is possible that the high activity of this antioxidant enzyme could be explained by a compensatory mechanism in an attempt to restore the appropriate redox state of DIO animals. In fact, the activity of this enzyme can increase or decrease in oxidative stress situations depending on the moment of their evolution and the amount of peroxides generated (Liu *et al*., 2004; Yan and Harding, 1997). Differences were also observed between the sexes in the redox parameters studied of middle-aged DIO animals, with males being more affected than females. Thus, in response to the late age of obesity onset, middle-aged male DIO mice showed higher values of xanthine oxidase and lipid peroxidation (MDA) and lower values of catalase activity in comparison with their female counterparts. Previous studies indicate that males are more susceptible to present higher oxidative stress than females (Baeza *et al*., 2011); however this situation is still controversial, and contradictory data have been reported (Kander *et al*., 2017). Moreover, there is a lack of evidence about the redox state of obese individuals comparing males and females.

At old age, female DIO mice continue to exhibit worse immune functions and redox state parameters than old female non-DIO controls. However, in contrast to our previous sub-objective

(in the early adulthood obesity onset), the late adulthood diet-induced obesity onset resulted in no significant differences in some immune functions (such as anti-tumour NK cell activity and basal proliferation of lymphocytes), redox state (xanthine oxidase activity, GSSG/GSH ratio, catalase and glutathione peroxidase activities) as well as in the life span between female DIO and non-DIO mice. Thus, the early adulthood obesity onset, in which adolescent mice were exposed to a high-fat diet, had more long-lasting deleterious effects on the immune system, and consequently on the life span than a later obesity onset. Importantly, increasing evidence suggests adolescence as a critical period in which the nervous and immune systems are still experiencing developmental changes and thus are more susceptible to stresses, such as an increased consumption of a high-fat diet (Boitard *et al*., 2012; Holder and Blaustein, 2014; Simon *et al*., 2015; Spear, 2000).

4.1.2. Effects of the blockage of the neonatal leptin surge (PND5-9) on the immune function and redox/inflammatory state in male and female rats at different ages

In the two first sub-objectives we studied **the effects of the blockage of the neonatal leptin surge (PND5-9) on the redox/inflammatory state and immune function in peripubertal/adolescent male and female rats,** and in the third sub-objective **we analysed the effects in adult rats.**

Similar to that described in mice (Ahima *et al*., 1998), rats also experience a plasma surge of leptin during the neonatal period (Delahaye *et al*., 2008). In these animals, the production of leptin increases between PND4 and 7, is elevated between PND7 and 10 and declines by PND14. Therefore, the present leptin antagonist treatment was administered precisely during the time of the leptin surge. Leptin appears to play a key role in immune and inflammatory responses (Conde *et al*., 2014; Valleau and Sullivan, 2014). However, the implication of the neonatal leptin surge in the development and establishment of these responses later in life has not been previously analysed.

The spleen is the largest secondary immune organ in the body and is responsible for initiating immune reactions to blood-borne antigens and for filtering the blood of foreign material and old or damaged red blood cells. In rats, this organ reaches peak development at puberty, followed by gradual involution. It appears to be a key player in cytokine production when there is an infection or after trauma resulting in systemic inflammation regulated by the autonomic nervous system (Gigliotti and Okusa, 2014). We found changes in the spleen weights of **peripubertal** animals treated neonatally with the leptin antagonist. The spleen of treated males weighed less and those of treated females weighed more than their controls. Since leptin acts as an immunomodulator (Conde *et al*., 2014; Valleau and Sullivan, 2014), it seemed plausible that the treatment affected the correct development of the spleen in both sexes. Neonatal leptin antagonist treatment clearly impaired the maturation of peripheral tissue such as pancreas, kidney, thymus and ovary in rats (Attig *et al*., 2011), so it is not surprising to find changes in spleen weight. Nevertheless, we were

not able to explain the mechanisms that resulted in these higher and lower spleen weights of males and females, respectively. However, these abnormal weights could indicate impaired development and function in the spleen of both sexes. Regarding body weight, we found no effect of the leptin antagonist treatment of either sex during neonatal and pre-pubertal life, as has been reported (Attig *et al*., 2008; Granado *et al*., 2011; Mela *et al*., 2012a; 2012b). As previously suggested (Mela *et al*., 2015), one possible explanation for no change in body weight, but a decrease in adipose tissue in males could be that there is an increase in lean body mass.

SOD acts as an enzymatic antioxidant defence that transforms the superoxide anion to H_2O_2 and works in parallel with CAT, which is responsible for the elimination of the excess of H_2O_2 (Vida *et al*., 2014). We found lower activities of both enzymes in the animals treated in the neonatal period with the leptin antagonist. These results are indicative of decreased antioxidant defences in the spleen. The glutathione system includes GPx and GR, two enzymes that play an important role in maintaining the correct balance between GSH and GSSG ratio. In normal conditions, glutathione is usually found in its reduced form since GR is constitutively active (Vida *et al*., 2014). In the present study, the treated animals showed higher GR activity and lower GPx activity than the corresponding controls. It is plausible that an increase in GR activity acts as a compensatory mechanism to mitigate the oxidative stress. In fact, when the ratio GSSG/GSH was analysed as a marker of oxidative stress, no significant effect of the neonatal treatment was found.

Appropriate concentrations of leptin are needed for a correct immune response (Stofkova, 2009). Leptin appears to stimulate both innate immunity, through the up-regulation of TLR expression in monocytes, and adaptive immune response carried out by lymphocytes. This stimulation is related with the protection against infections and associated with the production of pro-inflammatory cytokines and autoimmune diseases (Conde *et al*., 2014; Zabeau *et al*., 2014; Zarkesh-Esfahani *et al*., 2001). Indeed, both obese *ob/ob* (leptin deficient) and *db/db* (deficient in leptin receptors) mice had deteriorated immune responses and increased infection susceptibility (Milner and Beck, 2012) and leptin administration counteracts this impaired immune response in

animal models of leptin deficiency (Busso *et al*., 2002; Lord *et al*., 1998). In addition, leptin deficiency is associated with the decreased production of several cytokines. However, leptin regulation of cytokines is very different depending on several factors, notably the type of stimulus involved (Faggioni *et al*., 2000a; 2000b). Therefore, in order to measure the effects of the leptin antagonist on pro-inflammatory and anti-inflammatory cytokine secretion by spleen leukocytes, these cells were stimulated with either LPS or ConA. With both types of stimulation, we found lower IL-10 concentrations in spleen leukocytes from males and females that have been treated with the antagonist. Since IL-10 is clearly an anti-inflammatory cytokine, this observation suggests decreased protection against a potential inflammatory state. Moreover, lower concentrations of anti-inflammatory cytokines such as IL-10 have been detected in *ob/ob* mice after LPS treatment (Faggioni *et al*., 2000b). The concentrations of IL-13, a typical Th2 cytokine with anti-inflammatory and immunoregulatory actions (Huang *et al*., 2015; Wynn, 2015), were lower in LPS-stimulated leukocytes from males and in ConA-stimulated leukocytes from females. The pro-inflammatory cytokine TNF-α was lower in the LPS-stimulated leukocytes from males treated with the leptin antagonist, whereas the opposite was found in LPS-stimulated leukocytes from treated females, i.e. TNFα concentrations increased. In regards to this latter result, leptin has been shown to inhibit TNF-α induction by LPS in female mice. However, the methodology was different from our study, given that, in this study, female mice were 5 to 6 weeks old, and the LPS was administered intravenously (Faggioni *et al*., 2000a; 2000b). Some results suggest that a defect in leptin production is associated with a shift of the immune response toward a pro-inflammatory phenotype as a consequence of the up-regulation of pro-inflammatory and down-regulation of anti-inflammatory cytokines. However, no conclusive data are available on TNF concentrations with LPS, with unchanged, decreased or increased production of this cytokine having been reported (Faggioni *et al*., 2001). As a whole, the present data support the view that the pattern of cytokines regulated by leptin is very different depending on the type of stimulus used to induce their release (Faggioni *et al*., 2000a; 2000b). Moreover, we showed clear differences between males and

females. It is worth re-emphasizing that the males and females used in this study were of different chronological ages and, therefore, the differences observed between males and females may not be exclusively attributed to their sex. This does not negate the fact that blockage of neonatal leptin signalling affects the future oxidative status of the animals and their ability to respond to inflammatory insults during the critical period close to pubertal onset in both males and females.

When we analysed if there were changes in the mRNA of these antioxidants and cytokines in other tissues, we found significant effects in WAT. Males appear to be more affected by the treatment with leptin antagonist than females, as they present an increase in IL-1β and IL-6 and a decrease in CAT and GR mRNA concentrations. However, we found a decrease in TNF-α mRNA concentrations in females treated with the antagonist, corroborating the idea that the effect of this leptin antagonist is sex dependent. How these effects compromise the health status of individuals in the presence of an injury or immune challenge is unknown. Further investigation is needed to clarify the inflammatory state of these animals throughout their life. We previously found that maternal deprivation (MD) for 24 hours on postnatal day 9 (i.e., during the neonatal leptin surge period) produces a marked decrease in leptin concentrations (Viveros *et al*., 2010), as well as short- and long-term detrimental effects on the immune system (De la Fuente *et al*., 2009; Viveros *et al*., 2009). Thus, it is likely that both a social intervention such as MD and the present pharmacological treatment affect the development of the organism's defence system by interfering with the neonatal leptin surge. Therefore, in the second sub-objective we studied **the effects of the blockage of the neonatal leptin surge (PND5-9) on the function and inflammatory state in spleen leukocytes of peripubertal/adolescent male and female rats.** The results, as previously mentioned, also showed severe impairments in these parameters.

This study was only carried out in spleen since this secondary lymphoid organ, which is the largest in both humans and rodents, is responsible for mounting effective innate and adaptive immune responses and for regulating cytokine production (Cesta *et al*., 2006; Nolte *et al*., 2002). The animals treated with a specific leptin antagonist during the leptin surge (PND5-10) displayed

significantly impaired chemotactic capacity, anti-tumour NK activity and proliferation of lymphocytes in response to mitogens (LPS and ConA). Moreover, in cultures of those leukocytes under ConA and LPS-stimulated conditions, an impairment of the release of several cytokines was generaly shown in those rats treated with the leptin antagonist.

The immune system in early life goes through rapid and critical changes. Thus, perturbations in this period could result in long-lasting detrimental effects in later life (Goenka and Kollmann, 2015). In this sense, the neonatal programming of adult immune function has been previously reported. Thus, the neonatal exposure to an immune challenge, such as bacterial endotoxin LPS, has shown to alter neuroendocrine and immune responses in adult animals (Shanks *et al*., 1995; 2000; Spencer *et al*., 2001). Several mechanisms underlying these effects seem to be related to hypothalamic-pituitary-adrenal axis modifications (Spencer *et al*., 2001). In fact, a previous study in which rats were neonatally treated with the same leptin antagonist found long-term alterations on hypothalamic expression of reproductive and metabolic neuropeptides (Mela *et al*., 2016). Given the bidirectional communication between the nervous and immune systems, this alteration exerted on the hypothalamus could also have an impact on the immune system (Gomez-Nicola *et al*., 2013; Vida *et al*., 2014). In addition, although there is increasing evidence showing the effects of leptin on several immune system functions, no information is available specifically correlating the neonatal leptin surge with these functions at peripubertal age. For this, in the present sub-objective, the pegylated super leptin antagonist was administrated in male and female rats from PND5 to 9. This period of time is coincident, according with previous reports, to the plasma surge of leptin in neonatal mice and rats (Ahima *et al*., 1998; Delahaye *et al*., 2008).

With respect to the innate immunity, the functions studied were significantly impaired in spleen leukocytes of peripubertal male and female rats neonatally treated with leptin antagonist. Thus, the NK cells showed a lower lytic activity against tumour cells in treated animals than in controls. Therefore, it seems that the leptin surge has an important role in establishing and maintaining the proper function of NK cells. In fact, as previously reported leptin is involved in the

processes of NK cell development, differentiation, activation, and cytoxicity (Tian *et al*., 2002; Zhao *et al*., 2003). It was found that leptin enhances these processes in NK cells via the activator of transcription 3 (STAT-3) and by the up-regulation of the expression of IL-2 and perforin genes (Zhao *et al*., 2003). In relation to the activity of chemotaxis, this function was suppressed in cells from male and female treated animals. The role of leptin in the migration of immune cells has been observed given that it shows a chemoattractant capacity at low concentrations. This effect seems to be mediated via full-length leptin receptors and canonical migratory signalling pathways (Gruen *et al*., 2007). Moreover, leptin has been shown to stimulate the expression of chemokines in murine macrophages through the activation of the Janus kinase 2/signal transducers and the activators of transcription 3 (JAK2/STAT3) pathway (Kiguchi *et al*., 2009).

The neonatal administration of the leptin antagonist also resulted in impaired adaptive immunity in peripubertal rats. Thus, the proliferation of spleen lymphocytes in response to T- and B-cell mitogens, such as ConA and LPS, respectively, was lower in peripubertal female treated rats than in controls. An increased proliferation of lymphocytes has been observed in the presence of leptin (Lord *et al*., 1998). Enhanced proliferation and activation of human T lymphocytes with leptin were also observed when cells are co-stimulated with phytohaemagglutinin (PHA) or concanavalin A (ConA) (Martín-Romero *et al*., 2000). Moreover, leptin also plays an important role in the development of lymphocytes. In this sense, leptin receptor-deficient *db/db* mice showed impaired lymphopoiesis and reduced numbers of lymphocytes in peripheral blood (Bennett *et al*., 1996).

Increasing evidence suggests that leptin has a regulatory role within the cytokine network (Gainsford *et al*., 1996; Lam and Lu, 2007; Lord *et al*., 2006; Martín-Romero *et al*., 2000; Santos-Alvarez *et al*., 1999). Thus, in this study, in agreement with the impairments found in innate and adaptive immune functions, the neonatal leptin antagonist treatment also resulted, in general, in a lower release of several cytokines in ConA and LPS-stimulated spleen leukocytes of peripubertal rats. The IL-2, which is an essential regulatory cytokine that acts mainly on lymphoid populations,

including T, B, and NK cells (Liao *et al*., 2011), was found to be diminished in female neonatally treated animals. In this sense, leptin has been shown to influence the proliferation and secretion of IL-2 by immune cells, through the activation of the mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3-kinase (PI3-K) pathways (Liao *et al*., 2011; Lord *et al*., 1998). Since IL-2 is related to the proliferation of lymphocytes, the lower levels of this cytokine in treated females could explain the reduced lymphoproliferative response in the cells of this sex.

In addition, several studies showed that leptin signalling increased inflammatory mediators through direct activation of immune cells (Matarese *et al*., 2005; Procaccini *et al*., 2012; Tian *et al*., 2002). Thus, IL-1α and IL-1β, which are classic pro-inflammatory cytokines and are also involved in the proliferative response of lymphocytes (Zarkesk-Esfahani *et al*., 2001), exhibited lower concentrations in peribupertal male and female rats neonatally treated with leptin antagonist under LPS or ConA-stimulated conditions. Lower concentrations of IL-6 were also found in treated peripubertal male and female animals under mitogen (LPS or ConA) stimulation. Together with IL-2, IL-1α and IL-1β, IL-6 is crucial in the orchestration of the differentiation and function of innate and adaptive immune cells (Dinarello, 2009). These cytokines regulate T lymphocyte activation and differentiation (Garlanda *et al*., 2013; Sims and Smith, 2010). In addition, IL-1β and IL-6 promote B cell functions and antibody production (Dinarello, 2009; Fisher *et al*., 2014). Previous studies clearly demonstrated an association between the regulation of these cytokines and leptin. Thus, leptin has been shown to directly induce the release of IL-1β and IL-6 in microglia cells (Ataie-Kachoie *et al*., 2014; Tang *et al*., 2007) and to mediate the LPS-induced symptoms of sickness behaviour through a process that involves IL-1β induction in the brain (Lafrance *et al*., 2010; Sachot *et al*., 2004).

The levels of IL-12p70, a pro-inflammatory cytokine known to promote Th1 immune differentiation against intracellular pathogens, including bacteria and some viruses (Raphael *et al*., 2015), were lower in ConA or LPS-stimulated leukocytes from male and female treated rats. In addition, GM-CSF concentrations were diminished in LPS-stimulated leukocytes from treated

males and it should be borne in mind that this cytokine, which has been typically recognized as a haematopoietic growth factor, plays an important role in Th1 and Th17 responses (Shiomi and Usui, 2015). However, the concentrations of IFN-γ, also known as a Th1 cytokine with pro-inflammatory properties, were increased in ConA-stimulated leukocytes from treated males, whereas no differences were found in female treated rats. The previous sub-objective, in which the same experimental design was used, also found increased concentrations of TNF-α (which is also a Th1 cytokine with pro-inflammatory properties) in ConA-stimulated leukocytes from treated males. In addition, previous data indicate that, in several cases, a defect in leptin production could lead to an increase in the secretion of several pro-inflammatory cytokines, favouring the up-regulation of pro-inflammatory mediators (Faggioni *et al*., 2001). However, other studies using rodents with genetic abnormalities in leptin or leptin receptors showed suppressed expression of pro-inflammatory cytokines, such as IL-12 and IL-6, in response to LPS stimulation (Loffreda *et al*., 1998).

The release of T-helper type 2 (Th-2) cytokines, including the anti-inflammatory cytokines IL-4 and IL-5, which are commonly involved in the clearance of parasites and allergic reactions (Li *et al*., 2011), were reduced in LPS-stimulated leukocytes from male treated rats. However, no significant differences were found in female animals.

Our results suggest that the blockage of the neonatal leptin surge led to impairments both in Th1 and Th2 responses, although with a greater impact on Th1, since statistically significant differences were greater in Th1 cytokines, such as IL-2, IL-12p70 and IFN-γ, than in Th2 cytokines (IL-4 and IL-5). In this sense, previous data have shown that the reduction of leptin levels (commonly seen in conditions of malnutrition, anorexia nervosa or genetic leptin deficiency) resulted in impaired Th1 response and reduced immunocompetence as well as increased susceptibility to infection (Procaccini *et al*., 2012).

Given that previous studies showed the activation of JAK/STAT, MAPK and PI3K signalling pathways by leptin (Sánchez-Margalet *et al*., 2003), we could suggest a possible

involvement of these intracellular pathways in the alterations of immune and inflammatory responses of peripubertal rats neonatally treated with a leptin antagonist.

We have demonstrated in these previous studies that the neonatal disruption of the leptin surge resulted in impairments of immune functions, as well as of redox and inflammatory stress state during the peripubertal/adolescence period of rats. However, it seemed relevant to investigate whether **the blockage of the neonatal leptin surge (PND5-9) could produce these effects on immune function and redox state in adult male and female rats** (i.e., when the immune system has reached its complete development) (Simon *et al*., 2015). The results of the present sub-objective found that the exposure to the leptin antagonist from PND 5 to 9 resulted in a long-term impaired immune function and oxidative stress state in spleen leukocytes of adult male and female rats. The immune functions and oxidative state were also evaluated in the spleen, as in the previous studies.

With respect to the functions of the innate immunity analysed, both the chemotaxis and anti-tumour NK activities, were significantly impaired in spleen leukocytes of adult male and female rats treated with a leptin antagonist. However, the neonatal leptin antagonist treatment resulted in chemotactic response differences between peripubertal/adolescent and adult rats. Thus, adult treated rats showed a higher chemotactic response after peptide stimulation, whereas, in peripubertal/adolescent treated rats, this response was lower in comparison with their respective controls. The higher chemotactic response in adult treated rats could be interpreted as a possible compensatory mechanism, given that there is a lower response in other immune cell functions, such as the anti-tumour NK activity (Franceschi *et al*., 1995). In fact, the blockage of leptin in the neonatal period affected the NK activity so highly that not only did this function appear diminished at peripubertal age, but also at adult age. Previous studies demonstrated that leptin was able to modulate immune innate response, such as chemotaxis and NK cell activities. Therefore, as previously mentioned, leptin at low concentrations exhibited a chemoattractant capacity (Gruen *et al*., 2007) as well as to stimulating the expression of chemokines in immune cells (Kiguchi *et al*., 2009). In addition, leptin seems to modulate the NK cell activity via the activator of transcription 3

(STAT-3) and by the up-regulation of IL-2 and perforin genes (Zhao *et al*., 2003). The lack of leptin receptor expression in mice also results in impaired NK cell activity, which highlights the important role of leptin in regulating immune responses (Tian *et al*., 2002; Zhao *et al*., 2003).

The neonatal administration of the leptin antagonist also resulted in long-term impaired adaptive immune response in adult rats. In agreement with our previous data found in peripubertal rats, the proliferation of spleen lymphocytes in response to T- and B-cell mitogens, such as ConA and LPS, respectively, continued to be lower in adult male and female treated rats. Increasing evidence indicates that leptin is able to modulate immune functions of lymphocytes. For instance, leptin receptor deficient mice displayed impaired lymphopoiesis and a low number of lymphocytes in peripheral blood, which indicates that leptin is important for the development of lymphocytes (Bennett *et al*., 1996). In addition, an *in vitro* study demonstrated that leptin increased the proliferation of lymphocytes in the presence of mitogens (Martín-Romero *et al*., 2000).

In the present sub-objective, we found a long-term increased oxidative stress state in spleen leukocytes of adult treated rats. Thus, adult male and female rats neonatally treated with a leptin antagonist exhibited higher values of oxidant production, such as xanthine oxidase (XO) activity and GSSG contents, as well as lower concentrations of the antioxidant GSH and activities of its related antioxidant enzymes (GPx and GR) than those in the corresponding controls. Moreover, the GSSG/GSH ratios were higher, which showed increased oxidative stress (Asensi *et al*., 1999) and consequently the amount of MDA, a lipid peroxidation marker (Ayala *et al*., 2014), was also higher than in controls. These findings are partially in concordance with those found in the previous sub-objective of this thesis, which also evaluated a variety of oxidative stress parameters in the spleen leukocytes of peripubertal rats. Thus, in contrast to the results in adult rats, in which a lower activity of GR (an antioxidant enzyme) was observed, this activity was higher in treated peripubertal rats. The higher values of GR activity in treated peripubertal rats could be potentially explained as a compensatory mechanism in an attempt to the restore redox homeostasis in animals treated with leptin antagonist. Similarly, the aging process, which has been linked to increased oxidative stress

state, also described higher production of antioxidant enzymes as a compensatory mechanism (Rizvi and Maurya, 2007). Some studies have pointed to the possible involvement of leptin in modulating the oxidative stress response. Thus, in malnourished children with low amounts of leptin, enhanced lipid peroxidation as well as decreased antioxidant defence such as SOD and CAT activities as well as GSH concentrations were observed (Boşnak *et al*., 2010). Moreover, another study found that enhanced concentrations of leptin (in obesity and cardiovascular diseases) were also associated with an increased production of ROS. In this study, leptin seemed to increase ROS generation mainly by the activation of NADPH oxidase (Dong *et al*., 2006; Morawietz and Bornstein, 2006). Therefore, it appears that abnormal circulating concentrations of leptin, either diminished or enhanced, could contribute to the imbalance of redox homeostasis. It is curious that the decrease of leptin concentrations in the neonatal period allowed the maintenance of an oxidative stress in spleen at adult age. This is another aspect that should be studied in depth in the future.

4.1.3. Effects of the dietary supplementations with 2-OHOA or with the combination of n-3 fatty acids (EPA AND DHA) on immune function and redox state of adult female diet-induced obese mice, as well as on their life span

As we observed in the results of the first objective, DIO mice show an impaired function and a redox state in their immune cells. In the present objective we have shown that **the dietary supplementations with 2-OHOA or with the combination of n-3 fatty acid (EPA and DHA) improve immune function and redox state parameters in peritoneal leukocytes of adult female DIO mice, as well as their life span**. With respect to body weight, a long-lasting decrease in the body weight of 2-OHOA supplemented-DIO mice was observed during their aging process. Similarly, another study also found that 2-OHOA-treated rats experience a decrease in body weight through the induction of UCP-1 (uncoupling protein-1) expression in the adipose tissue, a process probably accompanied by enhanced energy expenditure (Vögler *et al*., 2008). The beneficial effects of 2-OHOA were also reflected in the life span of mice, and thus, although without statistical significance, the treated mice displayed an average survival (number of weeks) similar to control mice. Nevertheless, n-3 PUFA supplemented-DIO mice only showed significant improvements in immune and oxidative stress parameters, but no decrease in body weight or increase in life span. In that sense, evidence from a recent meta-analysis also indicated that supplementation with n-3 PUFA did not promote anti-obesity effects, such as reduction of weight, in overweight/obese individuals (Du *et al*., 2015).

The dietary supplementations with 2-OHOA or with the combination of EPA and DHA were able to revert the impaired macrophage functions of DIO mice, indicating a boost of the innate immune response towards pathogen destruction. Thus, DIO treated mice displayed improved chemotaxis, phagocytosis and generation of stimulated superoxide anion in peritoneal leukocytes. In agreement, findings from *in vitro* studies showed that neutrophils incubated with MUFA or with n-3 PUFA presented enhanced migration, phagocytic capacity and ROS production by immune

cells, suggesting immune-enhancing properties of fatty acids (Kumaratilake *et al*., 1997; Li *et al*., 1996; Padovese and Curi, 2009; Pisani *et al*., 2009). Several studies demonstrated that EPA and DHA could modulate immune functions in neutrophils of rats and humans, increasing chemotactic response, phagocytic activity and ROS production (Gorjão *et al*., 2007; Paschoal *et al*., 2013). In addition, administration of oleic acid induced neutrophil recruitment in a rat air-pouch model (Rodrigues *et al*., 2010) and mice fed a diet enriched with olive oil (MUFA) exhibited increased phagocytic activity in peritoneal macrophages (De Pablo *et al*., 1998b).

The anti-tumour NK activity of peritoneal leukocytes, which was shown to be impaired in DIO mice in the previous sub-objectives of this thesis and in other studies (De la Fuente and De Castro, 2012; Lamas *et al*., 2004) tended to be higher after supplementation with n-3 PUFA. Previous work demonstrated that ingestion of n-3 PUFA was able to improve NK cell cytotoxicity in sedentary rats (Robinson and Field, 1998). However, supplementation with MUFA did not lead to significant differences in the NK cell activity of DIO mice. Similarly, another study also did not find differences in the activity of NK cells in middle-aged men supplemented with a MUFA-rich diet (Yaqoob *et al*., 1998).

The lymphocyte functions, such as migration and proliferation in response to T cell-specific mitogen (ConA) or B cell-specific mitogen (LPS), showed lower values in DIO mice than in non-obese mice. However, the dietary supplementations used led to higher values for these functions. In this sense, an increased resistance to infection was found associated with enhanced proliferation of peritoneal cells under LPS stimulation in mice fed fish oil, rich in EPA and DHA, for 6 weeks (Blok *et al*., 1992). An improvement in the production of circulating IgM in response to an antigen was obtained after administration of a n-3 PUFA-rich diet in obese mice (Teague *et al*., 2013). Another study, in which mice were fed an olive oil-rich diet, exhibited slightly increased splenic lymphocyte proliferation (De Pablo *et al*., 1998a). Nevertheless, some animal studies have described an inhibition of lymphocyte proliferation with MUFA supplementation (Yaqoob *et al*., 1994a), whereas human studies have shown no changes (Yaqoob *et al*., 1998). It is possible that these

contradictory results could be influenced by the doses supplemented in the diets. A study in this regard found that lower doses of oleic acid enhanced lymphocyte proliferation, while higher concentrations could result in inhibition of proliferation through an increase in apoptosis (Gorjão *et al*., 2007).

Oxidative stress, which is generated by an imbalance between antioxidants and oxidants, also seems to play a critical role in obesity and obesity-related diseases (Grattagliano *et al*., 2008). In fact, in the present study, DIO mice showed higher values of oxidant production, such as xanthine oxidase activity and lipid peroxidation (MDA), as well as lower values of antioxidants (catalase activity and total glutathione concentrations) than non-obese control mice. This indicates that these DIO mice seem to exhibit an augmented oxidative stress state in comparison with controls. However, the dietary supplementations with MUFA or n-3 PUFA appear to enhance endogenous antioxidant defences in immune cells, as both treatments resulted in increased catalase activity and GSH concentrations. In addition, n-3 PUFA supplementation led to a lower activity of xanthine oxidase, whereas dietary supplementation with MUFA, resulted in a diminished production of MDA in peritoneal immune cells. Thus, these findings suggest that EPA plus DHA as well as 2-OHOA have a role in improving redox state in obese mice. Moreover, 2-OHOA appears to be less susceptible to lipid peroxidation than n-3 fatty acids, since supplementation with n-3 did not significantly diminish MDA concentrations. In agreement, previous studies described that the supplementation with extra virgin olive oil, which has been attributed an antioxidant role mostly by its high content of oleic acid (Pérez-Martínez *et al*., 2011), diminished the lipid peroxidation in rats (El-Kholy *et al*., 2014; Musumeci *et al*., 2014). Supplementation with n-3 PUFA also exhibited antioxidant properties in rats (Abdou and Hassan, 2014) and in a mouse model (Patten *et al*., 2013).

Although we demonstrated in the previous sub-objective that the supplementations with monounsaturated (2-OHOA) and n-3 polyunsaturated (DHA plus EPA) fatty acids improved the functions and redox state of immune cells, the study of **the effect of these supplementations on**

the oxidative stress in the brain, liver, lungs and kidneys of diet-induced obese (DIO) mice seemed relevant.

Our current findings showed increased oxidative stress in the brain (left and right cerebral cortex and hypothalamus), liver, lungs and kidneys (renal medulla and cortex) of diet-induced obese (DIO) mice in comparison with non-DIO controls. Thus, these DIO animals showed lower antioxidant defences, such as CAT and GR activities, as well as higher production of oxidants, including XO activity, GSSG/GSH ratios and lipid peroxidation (MDA concentrations) than non-DIO mice. The increased oxidative stress seems to be associated with lipotoxicity in these organs, which is generated when triglycerides are inappropriately stored in non-adipose tissues. The excessive accumulation of intracellular triglycerides is known to reduce the efficacy of the electron transport chain, causing the release of ROS and the generation of oxidative damage (Savini *et al*., 2013). In agreement with our current results, previous reports also indicate that obesity increased oxidative stress in a variety of organs, such as the cerebral cortex (Freeman and Keller, 2012), liver and kidneys (Noeman *et al*., 2011; Yuzefovych *et al*., 2013). These studies suggest that oxidative stress was related to adiposity, lipotoxicity, mitochondrial dysfunction and endoplasmic reticulum stress in a variety of organs from obese rodents (Freeman and Keller, 2012; Noeman *et al*., 2011; Yuzefovych *et al*., 2013). Moreover, another study showed that high-fat diet feeding increased mitochondrial hydrogen peroxide (H_2O_2) production, causing abnormal expression of antioxidant enzymes (such as CAT) (Rindler *et al*., 2013). Interestingly, we found higher values of glutathione peroxidase (GPx) activity (an antioxidant enzyme) in the liver, lungs and kidneys of DIO mice than in that of non-DIO mice. However, lower values of GPx activity were observed in the brain (right cerebral cortex) of DIO mice in comparison with non-DIO mice. The higher activity of this antioxidant enzyme could possibly be explained as a compensatory mechanism to protect these cells against oxidative damage (Vincent *et al*., 2001). The activity of this enzyme has been reported to increase or decrease in response to oxidative damage depending on the moment of its evolution and the amount of peroxides generated (Liu et al., 2004; Yan and Harding, 1997).

In turn, the dietary supplementations with 2-OHOA or with the combination of EPA and DHA in general were able to avoid the oxidative stress in the brain (with higher values of GPx and GR activities and lower values of MDA concentration) as well as in the liver, lungs and kidneys of DIO mice (with higher values of CAT and GR activities and lower values of XO activity, GSSG/GSH ratio and MDA concentration) in comparison with non-supplemented DIO mice. In all cases, the values showed similar values to those of the non-DIO controls. In agreement, our previous sub-objective also showed amelioration of oxidative stress after supplementation with 2-OHOA or n-3 PUFA (EPA and DHA) (with increased values of CAT and total glutathione concentration and decreased values of XO activity, GSSG/GSH ratio and MDA concentration) in peritoneal leukocytes of adult female DIO mice. Furthermore, another study demonstrated that oleic acid exhibited an antioxidant activity in *Caenorhabditis elegans*, which was regulated by the forkhead transcription factor DAF-16/FOXO (forkhead box protein O) (Wei *et al*., 2016). Other studies also showed the antioxidant properties of n-3 PUFA against lead acetate-induced toxicity in the liver and kidneys of rats (Abdou and Hassan, 2014) as well as in the brain of rats exposed to ethanol during gestation (Patten *et al*., 2013). One of the possible mechanisms involved in these effects could be the incorporation of unsaturated fatty acids into the cell membranes, which occurs in all tissues of the body, following their consumption. In addition, these fatty acids are known to modulate cell functions, by modifying membrane fluidity, lipid peroxide formation, eicosanoid production and gene regulation (De Pablo and Alvarez de Cienfuegos, 2000; Surette, 2008).

Interestingly, the supplementation with 2-OHOA did not improve the activity of GR and the ratio of GSSG/GSH in the liver of DIO mice. In addition, the supplementation with n-3 PUFA (EPA and DHA) increased the lipid peroxidation in the liver of DIO mice compared to non-supplemented DIO mice. These findings suggest that both supplementations, in particular with n-3 PUFA, were less effective in ameliorating the redox state in the liver in comparison with the other organs of DIO animals. This could possibly be explained by the central role of liver in the metabolism of fatty acids and by fact that PUFA supplemented DIO mice received a higher dose

(3000 mg of EPA and DHA/Kg HFD) than that of MUFA supplemented DIO mice (1500 mg of 2-OHOA/Kg HFD). Thus, it is possible that the lipototoxity and oxidative stress generated in the liver as a result of obesity could not be completely reversed by these supplementations. Similarly, our findings in the previous sub-objective also showed higher susceptibility to lipid peroxidation in peritoneal leukocytes of n-3 PUFA supplemented DIO mice than of MUFA supplemented DIO mice.

4.1.4. Effects of the dietary supplementation with fermented milk containing probiotics for different periods of time on behaviour, immune function and redox state in old mice, as well as on their life span

When **the effects of the dietary supplementation with fermented milk containing probiotics for one and four weeks on behaviour and immune function, as well as on the life span old mice, were analysed**, the results showed that a short-term (one week) supplementation was able to improve behaviour and immune cell functions of old mice, reaching similar values to those found in the adult controls. Furthermore, a longer-term supplementation (four weeks) maintained these improvements in the same parameters of immune functions. Nevertheless, the consumption of fermented milk with probiotics did not produce significant differences in the life span of mice. In this sense, it is possible that this consumption at old age was not sufficient to extend the life span, although the benefits found on behaviour and immune response indicate that this fermented milk with probiotics could allow a healthy aging. Similar results were obtained with other lifestyle strategies, such as the environmental enrichment, which started in old and adult age improved these parameters of health, but only was able to increase longevity in the latter (Arranz *et al*., 2010b)

The battery of behavioural tests, which evaluated the abilities of motor coordination, equilibrium, muscular vigour, exploratory activity and anxiety-like behaviour, was significantly impaired in old mice in comparison with adult mice. However, the one-week supplementation of fermented milk with probiotics was able to improve the performance of these behavioural parameters with respect to old non-supplemented controls. Thus, those animals displayed improvements in motor coordination and equilibrium (assessed by the wood rod test) and in muscular vigour (assessed by the tightrope test). There was also significantly increased exploratory activity, given that old mice supplemented with fermented milk displayed higher percentage of rearings and total number of head-dips in the holeboard test in comparison with old non-

supplemented mice. Moreover, they performed higher locomotion in the central area of the holeboard, which indicate higher exploratory activity and lower anxiety-like behaviour. Previous reports showed that mice tend to stay in the peripheral area (close the walls) and avoid the central area of the holeboard, which is considered more threatening for these animals (Lipkind *et al*., 1985; Simon *et al*., 1994). In addition, the self-grooming behaviour and the presence of defecation, which is usually considered an index of anxiety in mice (Archer, 1973; Kalueff *et al*., 2016), were significantly lower in old mice supplemented with fermented milk in comparison with old non-supplemented mice. Currently, there is little investigation about the effect of probiotics on behaviour of old individuals, given that most of the studies were performed in adults. In these studies, an association was found between the supplementation of probiotics and brain function and behaviour. Thus, Lactobacilli (latic acid bacteria) treatment for two months affected motor behaviour and decreased astrocyte reactivity of normal growing rats (Ushakova *et al*., 2009). Also, the ingestion of probiotics *Lactobacillus and Bifidobacterium* strains was associated with decreased anxiety in adult mice (Bravo et al., 2011; Savignac et al., 2014). There is also some evidence in humans that probiotics could affect mood and cognition (Messaoudi *et al*., 2011). The mechanisms by which probiotics affect brain function and behaviour remain unclear, but some evidence suggests their association with microbial composition changes, immune activation and production of neurometabolites (Cryan and Dinan, 2012).

The fermented milk with probiotics supplementation for one and four weeks was also able to improve macrophage functions of old mice, indicating immune-enhancing proprieties of probiotics. Thus, old mice supplemented with fermented milk displayed enhanced directed migration (chemotaxis) and phagocytic capacity of peritoneal macrophages in comparison with non-supplemented old mice. In agreement, another study demonstrated that the supplementation with fermented milk containing yogurt cultures and the probiotic bacteria *L. casei* DN-114001 for 14 days led to increased phagocytic activity in peritoneal macrophages of young mice (de Moreno de LeBlanc *et al*., 2008). In addition, a human-based study showed that the supplementation with the

probiotic *Bifidobacterium lactis* Bi-07 was capable of enhancing phagocytosis of monocytes and granulocytes in healthy elderly individuals (Maneerat *et al*., 2013).

The NK cells, known by their ability to recognize and kill tumour cells, showed decreased activity in old animals. However, the supplementation with fermented milk was able to improve this immune function in old mice after one and four weeks of supplementation. Previous studies in old individuals confirmed these results. Thus, a study showed that the ingestion of a fermented drink containing *Lactobacillus casei* Shirota for four weeks improved the NK cytotoxicity of healthy aged people (Dong *et al*., 2013). Moreover, another report indicated anti-tumour effects of fermented milk with probiotics consumption (Ohashi *et al*., 2002). This anti-tumour activity of probiotics seems to be the result of an improved immune response in the host (specially due to improvement of NK cell activity). Moreover, the consumption of probiotics appears to favour the reduction of the formation of carcinogenic and mutagenic compounds (Ohashi *et al*., 2002).

The lymphocyte functions, such as migration and proliferation in response to T cell-specific mitogen (ConA) or B cell-specific mitogen (LPS), showed decreased values in old mice than in adult mice. However, supplementation (one and four weeks) of fermented milk with probiotics led to improvements in the migration of lymphocytes. With respect to proliferation of lymphocytes in response to mitogens ConA and LPS, only a long-term supplementation (4 weeks) with probiotics increased this function in old mice. Others reports also indicated that probiotics ingestion could increase the response of specific antibodies against infection (Shu and Gill, 2001; Yasui *et al*., 1999).

The positive effects of the ingestion of fermented milk with probiotics in these immune functions studied, seems to be due to the direct action of probiotics. Thus, in the experiment carried out *in vitro*, the fermented milk supernatant (containing probiotics) enhanced chemotaxis activity and even showed a higher chemoattractant capacity. Moreover, this milk supernatant also stimulated the activity of NK cells.

Interestingly, although with fewer improvements than that with supplementation with probiotics, the control group of old mice which were supplemented with skimmed milk enriched with vitamins B6 and D also exhibited some benefits in behaviour and immune cell function parameters (specially after four weeks of supplementation). These results could be expected considering that these vitamins are known by their action in the nervous and immune functions (Dakshinamurti and Dakshinamurti, 2015; Prietl *et al*., 2013; Rail and Meydani, 1993; Wrzosed *et al*., 2013).

Since in the previous sub-objective the effects of the supplementation of fermented milk with probiotics on redox parameters had not been studied, and neither the effects of two weeks of this supplementation on behaviour and immune functions of peritoneal leukocytes, in this second sub-objective we investigated **the changes in several parameters of oxidative stress in peritoneal leukocytes, together with functions of these cells and behaviour response in old mice after the supplementation with a fermented milk drink for the period of two weeks.** In addition, we analysed the body weight of these animals. The results showed that the supplementation with fermented milk containing probiotics for two weeks was able to improve behaviour as well as the redox state and functions of peritoneal leukocytes in old mice. Moreover, during the supplementation period, there were significant differences in the body weight between old mice supplemented with fermented milk (probiotics) and non-supplemented mice. Thus, old supplemented mice maintained a similar body weight during this period of two weeks, whereas old non-supplemented mice had a significant loss of body weight. In this sense, it is known that the maintenance of a constant body weight during advanced aging is considered a positive health outcome, since the age-related body weight loss is often associated with malnutrition and sarcopenia, as well as is one of the parameter characteristic of frailty (Janssen *et al*., 2002; Keller and Engelhardt, 2014; Martínez de Toda *et al*., 2018). In fact, malnutrition, sarcopenia and frailty have been associated with increased risk of morbidity and mortality in old age (Janssen *et al*., 2002; Keller and Engelhardt, 2014; Martínez de Toda *et al*., 2018). Nevertheless, the consumption of

fermented milk with probiotics did not result in significant differences in the life span of mice, similarly to that observed when mice were supplemented with this fermented milk drink for the period of four weeks. Thus, it is possible that the supplementation with fermented milk at old age was not sufficient to extend the life span of mice, although the benefits found on behaviour, redox state and immune functions could indicate a healthy aging. Another strategy of lifestyle, such as the environment enrichment, has also shown an improvement of the redox state and immune functions in old mice without a significant effect on longevity, as previously mentioned. However, when this strategy was applied from adult age, there was a significant increase of the life span (Arranz *et al*., 2010b).

With aging, there is a progressive deterioration of brain and behavioural functions, such as muscular vigour, motor coordination, and equilibrium (Schulz *et al*., 2002; Seidler *et al*., 2010). In addition, old individuals are more likely to suffer from mental disorders, such as anxiety (Flint, 1994). In the present study, the supplementation with fermented milk for two weeks was able to restore this impaired behavioural response of old mice. Thus, several behavioural parameters, such as muscular vigour (assessed by the tightrope test), vertical exploratory activity (assessed by the performance of rearings) and horizontal exploratory activity (assessed by the ambulation of mice in the holeboard test) were improved in old mice supplemented with fermented milk. In addition, these supplemented animals showed a higher central exploratory activity in the holeboard. In this sense, previous studies indicated that the central ambulation was associated with lower anxiety-like behaviour, given that mice prefer to stay in the peripheral area (close to the walls), avoiding the central area (which is considered more threatening for mice) (Lipkind *et al*., 2004; Simon *et al*., 1994). Moreover, the behaviour of repeated digging and the presence of defecation, which may be considered anxiety-related measures (Archer, 1973; Saka and Graybiel, 2003), were significantly lower in old mice supplemented with fermented milk than in non-supplemented mice. Nevertheless, the performance of self-grooming, which is usually considered an anxiety-like behaviour (Kalueff *et al*., 2016), was higher in old supplemented animals than in non-supplemented controls. In this

regard, previous studies suggest that the self-grooming behaviour could be elicited by both opposite conditions, such as comfort and stress (Kalueff and Tuohimaa, 2004). Thus, it is possible that the higher performance of self-grooming in old supplemented mice could indicate comfortable, rather than stressful situations. These results are in agreement with those previously found in old mice supplemented with the same fermented milk for one week. Thus, a longer time of supplementation (two weeks) maintained these improvements. In addition, other studies performed with different probiotic strains (*Lactobacillus plantarum*, *L. fermentum*, *L. rhamnosus*, *L. helveticus* R0052, *Bifidobacterium longum* 1714, *B. breve* 1205 and *B. longum* R0175) have also shown their positive effects on behaviour, including improved motor coordination and reduced anxiety-like behaviour, in young and adult rodents (Bravo *et al*., 2011; Messaoudi *et al*., 2011; Savignac *et al*., 2014; Ushakova *et al*., 2009). In this sense, current evidence indicates that the gut microbiota and the administration of probiotics could regulate behaviour and functions of the central nervous system through neuroimmune and neuroendocrine mechanisms, which mostly involving the vagus nerve (Bravo *et al*., 2011). This communication seems to be mediated by microbially derived molecules, including short-chain fatty acids, secondary bile acids, and tryptophan metabolites (Yano *et al*., 2015; Samuel *et al*., 2008). These molecules appear to interact primarily with enteroendocrine cells, enterochromaffin cells and the mucosal immune system, but also could cross the intestinal barrier, enter systemic circulation, and reach brain sites (Martin *et al*., 2018).

In the present sub-objective, we found higher activities of CAT and GR as well as a lower concentration of oxidized glutathione (GSSG) in peritoneal leukocytes of old mice supplemented with fermented milk in comparison with non-supplemented old mice. These results are indicative of lower oxidative stress in the supplemented old animals than in the non-supplemented old animals. In this sense, increasing evidence suggests that certain strains of lactobacilli and bifidobacteria have antioxidant properties both *in vivo* and *in vitro*, and therefore could decrease oxidative stress (Kullisaar *et al*., 2002; Lin and Yen, 1999; Mikelsaar and Zilmer, 2009; Shen *et al*., 2011; Spyropoulos *et al*., 2011; Zanoni *et al*., 2008). In particular, a previous study showed that the

supplementation of fermented milk with *Lactobacillus fermentum* for two months enhanced the activity of several antioxidant enzymes (superoxide dismutase, catalase and glutathione peroxidase) in old mice (Sharma *et al*., 2014b). The mechanisms of the antioxidant action of probiotics are largely unknown, however, some evidence suggests that probiotics may modulate the redox status of the host via their metal ion chelating ability, ROS scavenging, enzyme inhibition, and regulation of signalling pathways (Wang *et al*., 2017).

With respect to the functions of immune cells, the present supplementation was also able to improve age-related impairments of macrophage functions in old mice (Martínez de Toda *et al*., 2016). Thus, these supplemented animals displayed enhanced migration (chemotaxis) and phagocytic capacity of peritoneal macrophages in comparison with non-supplemented mice. In agreement, our previous findings also showed immune-enhancing proprieties after the consumption of the same fermented milk drink for one week. Another study, which also used the same fermented milk drink, demonstrated that this supplementation, in young mice, resulted in increased phagocytic activity of peritoneal macrophages (de Moreno de Le Blanc *et al*., 2008). In addition, the supplementation with the probiotic *Bifidobacterium lactis* Bi-07 or *Lactobacillus fermentum,* in aged humans and mice, respectively*,* was able to enhance the phagocytosis of monocytes and neutrophils (Maneerat *et al*., 2013; Sharma *et al*., 2014b).

With aging, the anti-tumour activity of NK cells was impaired (Martínez de Toda *et al*., 2016). However, the supplementation with fermented milk for two weeks was able to enhance the values of this immune function in old supplemented mice in comparison with non-supplemented mice. In agreement, our previous results showed higher anti-tumour NK activity after one and four weeks of supplementation than in the controls. Similarly, the ingestion of a commercial fermented drink containing *Lactobacillus casei* Shirota for four weeks increased the NK activity of healthy aged people (Dong *et al*., 2013).

The functions carried out by lymphocytes, such as migration and proliferation in response to T cell-specific mitogen (ConA) or B cell-specific mitogen (LPS), are known to suffer age-related

impairments (Martínez de Toda *et al*., 2016). The supplementation with fermented milk, however, was able to improve these functions in old mice. Although these results are in agreement with those found in the long-term supplementation (four weeks) as we observed in our previous work, the short-term supplementation (one week) was not able to improve the mitogen-stimulated lymphocyte proliferation. Thus, it seems that this immune response of lymphocytes, which is considered a typical function of the adaptive immunity, were only improved after a period of supplementation of at least two weeks.

4.2. GENERAL DISCUSSION

4.2.1. Diet-induced obesity as a model of premature and accelerated aging

According to the oxidative and inflammatory theory of aging, the generation of chronic oxidative stress and inflammatory stress is the main cause of aging, affecting especially the regulatory systems. This theory also proposes the immune system as a critical player in these stresses, and consequently in the rate of aging (De la Fuente and Miquel, 2009). Thus, as previously mentioned in this thesis, several immune function and redox parameters have been suggested as markers of health, rate of aging and predictors of longevity (De la Fuente and Miquel, 2009; Martínez de Toda *et al*., 2016; 2019).

Animal models have been used to study the process of aging, which although shorter, essentially mimics that of humans (Mitchell *et al*., 2015). There are several animal models of premature aging that have been suggested by our research group, such as ovariectomised rats and mice, tyrosine hydroxylase haploinsufficient mice, obese animals, and anxious mice. These animal models showed premature/accelerated immunosenescence, chronic oxidative and inflammatory stresses, and consequently a shorter life span (Baeza *et al.,* 2011; De Castro, 2016; De la Fuente, 2010; 2018a; De la Fuente and De Castro, 2012; Garrido *et al*., 2018).

The results obtained in the present thesis indicate diet-induced obesity as a model of premature and accelerated aging. On one hand, the early adulthood diet-induced obesity onset, in which adolescent female mice were exposed to a high-fat diet, resulted in impaired immune functions, such as chemotaxis of both macrophages and lymphocytes, phagocytosis of macrophages, anti-tumour NK activity, mitogen-stimulated lymphoproliferation, and mitogen-stimulated release of several cytokines (IL-1β, TNF-α, IL-6, IL-2, and IL-10), as well as increased oxidative stress. These functions and redox state in peritoneal leukocytes of adult female diet-induced obese (DIO) mice showed similar values to those of chronologically old female animals.

Thus, these results confirm the state of premature immunosenecence and oxidative stress as a consequence of the early adulthood obesity onset. In addition, these DIO animals could be considered as prematurely aging, since they reached adulthood (which is when aging begins) with those characteristics of chronologically old mice. On the other hand, the late adulthood diet-induced obesity onset, in which late-adult male and female mice were exposed to a high-fat diet, resulted in impaired behaviour (such as low exploratory activity and high anxiety-like behaviour), deteriorated immune functions, including chemotaxis of both macrophages and lymphocytes, phagocytosis of macrophages, anti-tumour NK activity, basal and mitogen-stimulated lymphoproliferation, as well as increased oxidative stress. Some of these functions and redox state in peritoneal leukocytes of middle-aged female DIO mice showed values similar to those of chronologically old female animals. Thus, these results confirm the state of accelerated immunosenescence as a consequence of the late adulthood diet-induced obesity onset. These DIO animals could be considered as an accelerated aging model.

In addition, adult female DIO mice with premature immunosenescence (as a consequence of the early obesity onset) reached old age with aggravated immunosenescence, and consequently lived shorter than female non-DIO mice. However, differently from the early obesity onset, the late obesity onset resulted in no significant differences in some immune function parameters (such as the anti-tumour NK activity and LPS-stimulated proliferation of lymphocytes), redox state (xanthine oxidase activity, GSSG/GSH ratios, catalase and glutathione peroxidase activities) and in the life span between female DIO mice and female non-DIO mice. Therefore, there were differences in the age of obesity onset, with the early adulthood obesity onset resulting in more long-lasting deleterious effects in the immune system, and consequently in the life span than a late obesity onset. In this regard, increasing evidence suggest adolescence as a critical period in which the nervous and immune system are still experiencing developmental changes, and thus are more susceptible to stresses, such as an increased consumption of high-fat diet (Boitard *et al*. 2012; Holder and Blaustein, 2014; Simon *et al*., 2015; Spear, 2000).

4.2.2. Differences between males and females in the nervous and immune systems resulting from the late adulthood diet-induced obesity

There is increasing recognition that males and females show differences in the function of regulatory systems, and consequently in disease susceptibility and life span (Klein and Flanagan, 2016; Zagni et al., 2016). In the present thesis, we have demonstrated differences between males and femlaes in the body fat mass, biochemical parameters, behaviour, immune function and redox parameters, as a consequence of the late adulthood diet-induced obesity. In particular, middle-aged male DIO mice exhibited greater impairments in these parameters and functions, and consequently showed a shorter life span than their female counterparts.

It is largely accepted that there are differences in the body fat distribution between males and females, which have as major determinant the sexual steroids (Zore *et al*., 2018). Thus, males and oestrogen-deficient post-menopausal females tend to accumulate more abdominal and visceral fat and premenopausal females more lower body (gluteo-femoral) fat (Lovejoy *et al*., 2009). The visceral fat has been associated with increased occurrence of cardiovascular diseases, hyperlipidaemia, insulin resistance, and hypertension (Karastergiou *et al*., 2012). A recent study found that these differences in the effect of diet-induced obesity of mice depended on the age of onset. Thus, in juvenile mice (aged 18 weeks), the percentage of body weight gain was higher and the glucose tolerance was greater impaired in males than in females. However, in middle-aged mice (aged 44 weeks), the difference between males and females was reversed, and females gained substantially more weight and had greater impairment in glucose tolerance than males. This study also suggests that these differences in obesity could be influenced by sexual steroids, particularly oestrogen (Salinero *et al*., 2018). In a similar way, we also found that middle-aged female DIO mice, as a result of the late adulthood diet-induced obesity, displayed a higher total body fat mass than middle-aged male DIO mice. However, in contrast to this study, we found that middle-aged male DIO mice exhibited higher plasma levels of glucose and total cholesterol, indicating a worse

metabolic profile, than their female counterparts. In this regard, it is possible that the difference between the ages of diet-induced obesity onset (44 weeks vs 42 weeks) could have resulted in the changes of metabolic parameters. Moreover, this study used C57BL/6J mice, while we used ICR/CD1 mice.

Regarding behaviour, immune function and redox state parameters, males showed greater impairments than females, as a result of the late onset of obesity. In agreement, previous studies showed males more vulnerable than females to high-fat diet (HFD)-induced impairments, such as learning functions and synaptic plasticity (Hwang *et al*., 2010). In addition, males exhibited a lower innate immune response against infection in comparison with females, which could be further exacerbated by obesity (Jaillon *et al*., 2017). In obesity, there was an increased adipose tissue infiltration of M1 macrophages in males, but of M2 macrophages in females, and higher levels of inflammatory mediators in males compared to females (Singer *et al*., 2015). Thus, these findings suggest an increased pro-inflammatory milieu in males that could contribute to the higher impairments found in the immune and nervous system functions of this sex in comparison with females.

In the context of diet-induced obesity, the present thesis highlights the importance of studying age and sex as biological variables. In addition, the present data provide important **evidence of diet-induced obesity as a model of premature and accelerated aging**.

As **limitations** of the present study we could point out the not inclusion of males in the experiments performed at old age. Also, other relevant aspects of immunosenescence, such as senescence-associated phenotype (SASP), immunophenotyping aging subsets, and intracellular signalling pathways, could be considered in future studies to confirm the premature and accelerated immunosenescence of obese individuals.

4.2.3. Programming effect of the neonatal leptin surge in the redox/inflammatory state and immune function of peripubertal and adult ages of rats.

We found that the neonatal leptin surge disruption resulted in impaired functions and redox/inflammatory state in the spleen of peripubertal and adult male and female rats. Thus, these animals exposed to a leptin antagonist from PND5 to 9, which is coincident with the neonatal leptin surge, displayed significantly impaired chemotactic capacity, anti-tumour NK activity, LPS and ConA-stimulated lymphoproliferation, as well as an altered redox/inflammatory state of spleen leukocytes. Additionally and confirming the bidirectional communication between the regulatory systems, namely the nervous, endocrine and immune systems, the hypothalamus and white adipose tissue also showed an altered redox/inflammatory state in peripubertal treated animals. Thus, these results point to a **possible programming effect of the neonatal leptin surge on the establishment and maintenance of immune function and redox/inflammatory state.** In addition, it is reasonable to assume that any disruption/blockage of the neonatal leptin surge could represent a risk factor for the proper maintenance of the immune system function as well as overall health in later life.

As **limitations** of the present study we could point out that males and females at peripubertal/adolescent age were euthanized at slightly different ages. These animals were the same as those used in a previous study that focused on the effects of the same leptin antagonist treatment on hypothalamic systems related to reproduction. In addition, there was a lack of exploration of possible mechanisms between the neonatal leptin disruption and the impairments found in the redox/inflammatory state and immune function. Although beyond the scope of the current objective, this subject should be considered in future studies.

4.2.4. Nutritional interventions in models of premature and chronological aging

Many lifestyle interventions, such as physical exercise, physical and mental activity through environmental enrichment, hormesis and nutrition, have been proposed to improve functions of immune cells, decreasing their oxidative and inflammatory stresses, in animal models of premature and chronological aging. These interventions have demonstrated to ameliorate immunosenescence and oxidative stress, and consequently to promote healthy aging (De la Fuente *et al*., 2011). In particular, nutrition, such as the consumption of antioxidant compounds showed to ameliorate the age-related changes of the immune system in premature and chronological aging, through the modulation of function and redox state of immune cells (Alvarado *et al*., 2006; De la Fuente *et al*., 2011). In addition, this nutritional intervention demonstrated positive effects on the nervous system (with treated animals showing a better response in behavioural tests than non-treated animals) (De la Fuente, 2010; De la Fuente *et al*., 1998).

The present thesis focuses on two different nutritional interventions (unsaturated fatty acids and probiotics) in animal models of premature and chronological aging, respectively. Diet-induced obesity was considered a model of premature aging, since adult diet-induced obese (DIO) mice, as a consequence of being fed with a high-fat diet during their adolescence, presented values of immune function and redox state similar to those of chronologically old female mice.

We found that the nutritional interventions with 2-hydroxyoleic acid (2-OHOA), a synthetic monounsaturated fatty acid (MUFA), or with the combination of n-3 fatty acids (polyunsaturated fatty acids of the n-3 series), namely eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), were able to revert impaired function and oxidative stress of peritoneal leukocytes in adult female diet-induced obese (DIO) mice. Furthermore, the nutritional intervention with 2-OHOA also resulted in a tendency to increase the longevity of mice. Additionally, these nutritional interventions showed to revert oxidative stress in the brain, liver, lungs and kidneys of adult female DIO mice. Thus, these results confirm that **the nutritional interventions with 2-OHOA or with the**

combination of EPA and DHA could contribute to the promotion of a healthy aging through amelioration of immune dysfunction and oxidative stress in adult female DIO mice.

In addition, we found that the nutritional intervention with fermented milk containing yogurt cultures plus *Lactobacillus casei* DN-114001 at different periods of time (one, two and four weeks) was able to improve behaviour, immune function and redox state in chronologically old female mice. Nevertheless, this nutritional intervention was not able to extend the life span of mice. Thus, it is possible that the supplementation with fermented milk at old age was not sufficient to extend the life span of mice, although the benefits found on behaviour, immune function and redox state could indicate a healthy aging. Therefore, these results confirm that **the nutritional intervention with fermented milk containing probiotics for different periods of time resulted in the amelioration of behavioural and immune dysfunctions as well as oxidative stress in old female mice, thus contributing to a healthy aging.**

As **limitations** of the present study we could point out the lack of exploration of putative mechanisms through which the dietary supplementations improved behaviour, immune function and redox state of premature and chronological aging mice. Although beyond the scope of the proposed research, this subject should be considered in future studies.

5. CONCLUSIONS

The following conclusions can be drawn from the results obtained in the present thesis.

Regarding the first objective "*To study the effects of diet-induced obesity onset on behaviour, immune function and redox/inflammatory state at different ages, as well as on the life span of mice*", the following conclusions can be drawn.

1. The early adulthood diet-induced obesity onset produces premature immunosenescence and oxidative-inflammatory stresses in adult female mice.
2. The early adulthood diet-induced obesity onset aggravates immunosenescence in old female mice.
3. The late adulthood diet-induced obesity onset produces accelerated immunosenescence, oxidative stress and impaired behaviour in middle-aged male and female mice.
4. The late adulthood diet-induced obesity onset affects more middle-aged males than middle-aged females in several parameters of behaviour, immune function and redox state.
5. The late adulthood diet-induced obesity partially aggravates immunosenescence in old female mice.

Regarding the second objective "*To study the effects of the blockage of the neonatal leptin surge (PND5-9) on the immune function and redox/inflammatory state of male and female rats at different ages*", the following conclusions can be drawn.

6. The blockage of the neonatal leptin surge produces an impaired redox/inflammatory state in the spleen, hypothalamus and white adipose tissue of peripubertal/adolescent male and female rats.
7. The blockage of the neonatal leptin surge produces impairments in the functions and the inflammatory state of spleen leukocytes of peripubertal/adolescent male and female rats.

8. The blockage of the neonatal leptin surge produces impairments in the functions and redox state of spleen leukocytes of adult male and female rats.

9. The neonatal leptin surge seems to have an important physiological role in the establishment and maintenance of an appropriate immune system functioning.

With regards to the third objective "*To study the effects of dietary supplementation with 2-OHOA or with the combination of n-3 fatty acids (EPA and DHA) on immune function and redox state of adult female diet-induced obese mice, as well as on their life span*", the following conclusions can be drawn.

10. The dietary supplementation with 2-OHOA or n-3 PUFA (EPA and DHA) improves functions and redox state of peritoneal leukocytes in diet-induced obese (DIO) female mice, bringing the values similar to those found in non-DIO mice.

11. The dietary supplementation with 2-OHOA or n-3 PUFA (EPA and DHA) improves redox state in the brain, liver, lungs and kidneys of DIO mice, bringing the values similar to those found in non-DIO mice.

12. The dietary supplementation with 2-OHOA or n-3 PUFA (EPA and DHA) could be an effective nutritional intervention to restore the immune response and redox state of DIO mice.

With regards to the fourth objective *"To study the effects of the dietary supplementation with fermented milk containing probiotics for different periods of time on behaviour, immune function and redox state of old mice, as well as on their life span",* the following conclusions can be drawn.

13. The dietary supplementation with fermented milk containing probiotics for one and four weeks improves behaviour and immune function of old female mice, bringing the values

similar to those found in adult female mice. The improvement of the immune functions seems to be due to the direct action of probiotics.

14. The dietary supplementation with fermented milk containing probiotics for two weeks improves behaviour, immune function and redox state of old female mice.

15. The dietary supplementation with fermented milk containing probiotics, even in a short period of time, seems to be a good nutritional intervention to improve behaviour as well as functions and redox state of peritoneal leukocytes in old female mice.

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7. ABBREVIATIONS

2-OHOA	2-hydroxyoleic acid
4-HNE	4-hydroxynonenal
AA	Arachidonic acid
AgRP	Agouti-related peptide
AMPK	5′ adenosine monophosphate-activated protein kinase
ASP	Acylation stimulating protein
ATM	Adipose tissue macrophages
BAT	Brown adipose tissue
BCR	B-cell receptor
BDNF	Brain-derived neurotrophic factor
BMI	Body mass index
CART	Cocaine- and amphetamine-regulated transcript
CAT	Catalase
CCL	Chemokine C-C motif ligand
CD	Cluster of differentiation
CH_2	Methylene group
CI	Chemotactic index
CLP	Common lymphoid progenitor
CLS	Crown-like structures
CMP	Common myeloid progenitor
CNS	Central nervous system
ConA	Concanavalin A
COX	Cyclooxygenase
Cu	Copper
CVD	Cardiovascular diseases
CXCL	C-X-X motif chemokine ligand

DAMP	Damage-associated molecular pattern
DC	Dendritic cell
DHA	Docosahexaenoic acid
DIO	Diet-induced obese mice
DNA	Deoxyribonucleic acid
DPA	Docosapentaenoic acid
ENS	Enteric nervous system
EPA	Eicosapentaenoic acid
ER	Endoplasmic reticulum
Fe	Iron
FFA	Free fatty acid
fMLP	Formylated peptide
GABA	gamma-aminobutyric acid
GLUT	Glucose transporter
GPx	Glutathione peroxidase
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GR	Glutathione reductase
GSH	Tripeptide gamma-glutamylcysteine or reduced glutathione
GSSG	Glutathione disulphide or oxidized glutathione
HDL	High-density lipoprotein
HFD	High fat diet
HNO_2	Nitrous acid
H_2O_2	Hydrogen peroxide
HPA	Hypothalamic pituitary adrenal
HSC	Haematopoietic stem cell
ICAM-1	Intercellular adhesion molecule 1

IFD	Intermediate-fat diet
IFN	Interferon
IκBβ	Inhibitor of kappa B kinase beta
IL	Interleukin
iNKT	Invariant Natural killer T
iNOS	Inducible nitric oxide synthase
JAK2	Janus kinase 2
JNK	c-Jun N-terminal kinase
LAB	Lactic acid bacteria
LDL	Low-density lipoprotein
LOO•	Lipid peroxyl
LOX	Lipoxygenase
LPS	Lipopolysaccharide
M	Macrophage
MALT	Mucosal-associated lymphoid tissue
MAPK	Mitogen-activated protein kinase
MCP-1	Macrophage and monocyte chemoattractant protein-1
MDA	Malondialdehyde
MHC	Histocompatibility complex
Mn	Manganese
MNC	Mononuclear cell
MPO	Myeloperoxidase
mRNA	Messenger ribonucleic acid
MSH	Melanocyte-stimulating hormone receptor
mtDNA	Mitochondrial deoxyribonucleic acid
mTOR	Mammalian target of rapamycin

mtROS	Mitochondrial respiratory chain
MUFA	Monounsaturated fatty acid
NADPH	Nicotinamide adenine dinucleotide phosphate oxidase, reduced form
NFκB	Nuclear factor kappa B
NK	Natural killer
NKT	Natural killer T
NLR	Nucleotide-binding oligomerization-domain protein-like receptor
NLRP3	Nucleotide-binding oligomerization-domain protein-like receptor family pyrin domain containing 3
NMN	Nicotinamide mononucleotide
NOD	Nucleotide-binding oligomerization-domain protein
NOS	Nitric oxide synthase
NO•	Nitric oxide
NO_3•	Nitrogen dioxide
NPY	Neuropeptide Y
nucDNA	Nuclear mitochondrial deoxyribonucleic acid
O_2	Molecular oxygen
O_2•$^-$	Superoxide anion
Ob-R	Leptin receptor
OH•	Hydroxyl anion
ONOO•	Peroxynitrite
OPN	Osteopontin
PAM	Prematurely aging mice
PAI-1	Plasminogen activator inhibitor
PE	Phagocytic efficacy
PECAM-1	Platelet endothelial cell adhesion-1

PGE2	Prostaglandin E2
PHA	Phytohaemagglutinin
PI	Phagocytic index
PI3K	Phosphatidylinositol 3-kinase
PMN	Polymorphonuclear leukocyte
PND	Postnatal day
POMC	Pro-opiomelanocortin
PRPP	Phosphoribosyl pyrophosphate
PUFA	Polyunsaturated fatty acid
PWS	Prader-Willi syndrome
R•	Free radical species
RBP4	Retinol-binding protein 4
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
RNA	Ribonucleic acid
SFA	Saturated fatty acid
SOD	Superoxide dismutase
STAT-3	Activator of transcription 3
SVF	Stroma-vascular fraction
TCR	T-cell receptor
Th	T helper
TLR	Toll-like receptor
TNF	Tumour necrosis factor
UCP	Uncoupling protein
VEGF	Vascular endothelial growth factor
VLA-2	Very late antigen 2

WAT	White adipose tissue
XO	Xanthine oxidase
Zn	Zinc